THE UNIVERSITY OF WARWICK

OBESITY AND PERI-IMPLANTATION ENDOMETRIUM

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Contents

I DEDICATE THIS THESIS

To my parents for making me who I am and for being my inspiration,

To my wife (Pushpa), for all the sacrifices you have made for me to progress and in taking care of our family,

To my brother (Veeresh) and sister (Suneetha), for your unconditional love which always has motivated me to set higher targets

and

To my lovely son (Hem Aditya), for providing me with a magic shield of love that always surround me.

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DECLARATION

I, Dr Harish Malappa Bhandari declare that this thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Medicine (MD). It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented was carried out by me except in the cases outlined below:

1. Clinical data from women who attended Implantation Clinic for Chapters 3 and 4 were collected by Professors Quenby and Brosens. I was involved in conceiving the idea and data analysis.
2. Immunohistochemistry: Most of the endometrial Biopsies were obtained by Professors Siobhan Quenby and Jan Brosens and some by me. The slides preparation and immunohistological staining was undertaken by Mr Sean James and his colleagues at the Tissue Bank. I understand the principles of IHC. Interpretation of slides, analysis of leucocyte density, data generation and statistical analysis were done by me.
3. The clonogenic assay was developed in collaboration with Dr Keisuke Murakami and the data for chapter 4 was generated with him. I recruited some women for the study, obtained endometrial biopsies and performed laboratory experiments. I performed the statistical analysis for this thesis.
4. In-vitro differentiation of endometrial stromal cells: the laboratory work was undertaken in collaboration with Ms Sabeeha Malek, Ms Madhuri Salker and Ms Emma Lucas who helped in generating the data.

LIST OF PUBLICATIONS AND PRESENTATIONS

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Bhandari HM, *James S, Brosens J, Quenby S, Tan BK.* '*Obesity, as determined by BMI, does not impact upon uterine natural killer cell density in the peri-implantation endometrium'. BJOG. June 2013, Vol 120/s1, 205-206.*

Planned peer-reviewed publications:

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Bhandari HM, *Tan BK, Brosens J, Quenby S.* '*Superfertility is more prevalent in obese women with recurrent miscarriage'.*

Bhandari HM, *Tan BK, Brosens J, Quenby S. 'Female obesity is associated with an increased risk of miscarriage of anembryonic pregnancies'.*

Bhandari HM*, Murakami K, James S, Salker M, Malek S, Lucas E, Brosens J, Quenby S, Tan BK. 'Impaired endometrial decidualisation: A mechanism for reproductive failures in obese women'.*

Oral Presentation:

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Book chapter:

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ABSTRACT

Obesity is a global health problem and the current available evidence from the literature suggests that obese women may suffer from a wide spectrum of reproductive complications. The current understanding of obesogenic effects on the peri-implantation endometrium is limited and has become an important research topic as the emerging clinical evidence from the published studies indicate the possible role of the endometrium.

The first part of this thesis addresses the clinical question of whether an early pregnancy outcome is affected by the body mass index and whether there is any difference in time taken to achieve pregnancy in obese women with recurrent miscarriage when compared to normal weight women. The results are in chapters 3 and 4, where we demonstrated that obese women were more likely to have miscarriage of empty gestational sac or anembryonic pregnancies. In recurrent miscarriage context, the obese women were more ‘super-fertile’ suggesting the possible loss of an endometrial ability to select normal from abnormal pregnancies.

The second part of this thesis provides an analysis of the peri-implantation endometrial stromal compartment in normal weight and obese women. In chapter 5, using immunohistochemical methods it was shown that there was no difference in the uterine natural killer cell and macrophage density in the peri-

implantation endometrium of different weight groups. This suggests that the endometrial dysfunction in obese women with reproductive failure does not appear to be immune cell mediated.

In chapter 6 it was shown that the clonogenecity of endometrial mesenchymal stem cells (W5C5+) was significantly negatively correlated with the BMI. The obese women had significantly reduced cloning efficiency of W5C5+ cells when compared to normal weight women, suggesting of a possible sub-optimal regenerative capacity of the endometrium in obese women.

Finally, chapter 7 showed a potential association between obesogenic environment and impaired stromal cell decidualisation. Using an in-vitro model, it was shown that there was no significant difference in the expression of decidualisation markers (PRL and IGFBP1) in the decidualising endometrial stromal cells from normal weight women when compared to high BMI women. However, when the stromal cells were decidualised in an artificial obesogenic environment, the PRL expression was significantly inhibited in the presence of supernatant from adipose tissue explants of obese women when compared to normal weight women.

In summary, the findings from my work have provided an understanding of the peri-implantation endometrium in obese women and evidence to suggest that the endometrial stromal function is possibly facilitated by metabolic influences.

ABBREVIATIONS

ART – Assisted Reproduction Technique

BFS – British Fertility Society

BMI – Body mass index

cAMP – Cyclic adenosine mono-phosphate

CD – Cluster of differentiation

CFU – Colony forming unit or clonogenecity

CI – Confidence Interval

COS – Controlled ovarian stimulation

eMSCs – Endometrial mesenchymal-like stem cells

ESCs – Endometrial stromal cells

ESHRE – European Society of Human Reproduction and Embryology

EVT - Extravillous trophoblast

FOXO1 - Forkhead box protein

FSH – Follicular stimulating hormone

GnRH - Gonadotrophin releasing hormone

HB-EGF - Heparin binding EGF-like growth factor

hCG – Human chorionic gonadotrophin

HLA-G - Human leukocyte antigen G

HMB – Heavy menstrual bleeding

ICSI – Intracytoplasmic Sperm Injection

IFN – Interferon

IL – Interleukin

IU – International Unit

IUI – In-Utero Insemination

IVF – In-Vitro Fertilization

LH – Luteinising hormone

MII – Metaphase II

MBL – Menstrual blood loss

MUC1 – Mucin1 cell surface associated

NHS – National Health Service

NICE – National Institute for Health and Care Excellence

OI – Ovulation Induction

OR – Odds Ratio

PCOS – Polycystic Ovary Syndrome

PDGF - Platelet derived growth factor

PRL – Prolactin

PROK 1 – Prokineticin 1

RCOG – Royal College of Obstetricians and Gynaecologists

RM – Recurrent Miscarriage

ROC curve – Receiver operating characteristic curve

SGK1 - Serine-threonine protein kinase

SHBG – Sex Hormone Binding Globulin

TGF – Transforming growth factor

TNF-α – Tumour necrosis factor – α

uNK cells – Uterine natural killer cells

WHO – world Health Organisation

WMD – Weighted Mean Difference

11βHSD1 - 11-β hydroxysteroid dehydrogenase type 1 enzyme

CHAPTER 1:

INTRODUCTION

1.1 OBESITY

1.1.1 Definition

Obesity, defined as an abnormal or excessive fat accumulation that may impair health, is a complex condition that has serious physical, psychological and social consequences for an individual (WHO Fact sheet, Mar 2013). The central origin of this excessive fat accumulation results from an energy imbalance that results when an excessive intake of calories exceeds calories expenditure.

It is difficult to directly measure body fat and its distribution. The body weight may not provide an accurate estimation of the fat stores in the body. Hence, other measurements such as body mass index (BMI), waist to hip ratio, waist circumference, skin fold thickness and bio-impedance are being used to evaluate and define obesity (Haththotuwa et al., 2013). BMI is calculated by dividing weight in kilogrammes by the square of the height in meters (kg/m^2). BMI is relatively easy to calculate, non-invasive, inexpensive and reproducible (National Obesity Observatory, 2009). The BMI increases as the amount of body fat increases and increases the subsequent risk of obesity related illness (National Heart Foundation 2007). Though BMI is not the ideal measure of body fat, it has been the most widely accepted measure to estimate normal weight, overweight or obese, as in most people it compares well with their body fat levels (National Obesity Observatory, 2009). It is considered that body fat

distribution is more relevant than the actual weight as the visceral fat is metabolically more active and is responsible for the increased metabolic and cardio-vascular risks (Lord and Wilkin, 2002). However, the true measures of body fat using bio-impedance are impractical or expensive to use at population level. The waist:hip ratio and waist circumference are more strongly associated with metabolic risks and long term illnesses and should be ideally be used to define obesity, but it may be difficult to measure these, especially in obese.

1.1.2 Extent of the problem

Obesity is a global health problem. It was once considered as a being associated with affluence, but now, it can virtually affect all socio-economic ranks and age groups. According to WHO global estimates in 2008 more than 1.4 billion adults aged 20 and older were overweight and about half a billion of them were obese - nearly 300 million women and over 200 million men (WHO Fact sheet updated March 2013). At least 2.8 million people die each year as a consequence of being overweight and obese (WHO 10 facts about obesity, 2013). The highest prevalence of obesity has been reported be over 75% in Samoa and Nauru whereas it is less than 5% in China, Japan, India and certain African nations (WHO Global database on body mass index, update January 2014). There has been a tremendous increase in the prevalence of obesity in England in the last two decades and it is estimated that around one in four adult is now classified as obese – 24% of men and 26% of women (Craig and Mindell, HSE, 2010).

Classification	BMI (kg/m^2)	
	Principle cut-off points	**Additional cut-off points**
Underweight	**< 18.50**	**< 18.50**
Severe thinness	< 16.00	< 16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal	**18.5 - 24.9**	**18.50 - 22.99**
		23.00 - 24.99
Overweight	**≥ 25.00**	**≥ 25.00**
Pre-obese	25.00 -29.99	25.00 - 27.49
		27.50 - 29.99
Obese	**≥ 30.00**	**≥ 30.00**
Obese Class I	30.00 – 34.99	30.00 – 32.49
		32.50 – 34.99
Obese Class II	35.00 – 39.99	35.00 – 37.49
		37.50 – 39.99
Obese Class III	≥ 40.00	≥ 40.00

Table I: The International Classification of adult underweight, overweight and obesity according to BMI (Source: WHO – Global database on body mass index, updated January 2014.)

1.1.3 Impact on health and resources

Obesity exerts adverse metabolic effects on blood pressure, insulin resistance, triglycerides and cholesterol. The risk of acquiring non communicable diseases such as cardiovascular diseases (ischaemic heart disease and cerebrovascular accidents), diabetes, musculoskeletal disorders (osteoarthritis) and cancers (endometrial, breast and colon) are higher in overweight and obese individuals (WHO Fact sheet updated March 2013). It causes significant burden on the health resources and in England, it is estimated that £5.1 billion per year is being spent on managing the effects directly caused by obesity (Craig and Mindell, HSE, 2010).). Considering its serious implications on health and resources, WHO in 1997, formally recognised obesity as a major public health problem and a 'global epidemic'.

1.2 Obesity and Female Reproductive Health

The effects of obesity on reproduction start in children. The onset of puberty and menarche is earlier in obese girls when compared to their normal weight peers (Must et al., 2005; Himes, 2006). Consequences of early sexual development can generate psychosexual strain in young girls and their families (Lash and Armstrong, 2009). In animal studies, it has been demonstrated that leptin injection triggers the onset of puberty (Ahima et al., 1997) and in leptin deficient adolescent girl with proven hypogonadotrophic hypogonadism it has

been shown that leptin substitution led to a rapid induction of gonadotropin secretion and menarche (von Schnurbein et al., 2012). It is possible that large volumes of adipose tissue in obese children generate increased leptin levels that trigger the onset of puberty. Also, the bioavailability of sex steroids is more in obese girls due to increased aromatization of androgens in the adipose tissue, which stimulates earlier puberty (Ahmed et al., 2009). The risks of developing hyperandrogenism and PCOS are more common in girls with an early onset of puberty (Ibanez et al., 2009).

Heavy menstrual bleeding (HMB) is defined as excessive menstrual blood loss (MBL) that affects a woman's physical, social, emotional or material quality of life (NICE, 2007). HMB, with a more objective definition of total MBL of 80 ml or more (Warner et al., 2004), is a source of considerable morbidity to women. There is a lack of evidence in the literature to establish the impact of increased BMI upon quantity of MBL among women with HMB. Dysfunctional uterine bleeding is more common in overweight women as a result of aromatization of androgen to oestrogen in adipose tissue and thus altering the oestrogen-progesterone ratio. This prolonged excessive oestrogen stimulates endometrial proliferation and overgrowth leading to bleeding at irregular intervals (Lash and Armstrong, 2009). Weight loss may restore menstrual regularity in these women by reduction in the peripheral conversion of androgen to oestrogen.

Pre-menopausal obese women appear to have increased risk of developing endometrial polyps that may be associated with abnormal uterine bleeding. In a

survey, obesity in combination with hypertension was found to be a significant risk factor for the development of endometrial polyps (Resolva et al., 1999). In women undergoing IVF, BMI was found to be an independent risk factor for the development of endometrial polyps (Onalan et al., 2009).

In 2006, ESHRE Capri Workshop Group looked at nutrition and female reproduction, and summarised the available evidence on the effects of obesity on contraception. They concluded that combined oral contraceptive pills, contraceptive patches, progesterone only pills, contraceptive implants and the use of tubal rings for female sterilisation were less effective in women with increased body weight (ESHRE Capri Workshop Group, 2006). Variations in steroid metabolism and distribution, sequestration of steroids in adipocytes and dilution of the steroids in a larger blood volume were thought to be responsible for the reduced effectiveness of hormonal contraceptives in obese women. However, a recent Cochrane Database Systematic Review looked at the data from 13 trials that included 49,712 women, for the effects of BMI on contraception. Out of the five reports that compared BMI groups, only one trial suggested that women with a BMI of more than 25 were at a greater pregnancy risk and the other four trials did not show significant difference. The authors concluded that evidence did not show an association of BMI and hormonal contraceptive efficacy, and that the evidence was limited to assess the effectiveness of any individual contraceptive method (Lopez et al., 2013).

There is a strong association between obesity and endometrial cancer (Kaaks et al., 2002). Chronic exposure of the endometrium to unopposed oestrogen associated with PCOS causes endometrial proliferation and potential malignant changes. Further to this, hyperinsulinaemia and hyperandrogenism associated with obesity decrease the availability of SHBG leading to increased bioavailability of oestrogen, which further accentuates the deleterious effects of oestrogen on the endometrium (Kulie at al., 2011). Obese women are more likely to have increased incidence and mortality from cervical cancer and this may partly be due to poor compliance of obese women for the cervical screening programme (Maruthur et al., 2009). Increased serum oestradiol levels are also responsible for the increased development of breast cancer in obese women (Kuhl, 2005).

1.2.1 Obesity and Female fertility

Hippocrates wrote: ‘People of such constitution cannot be prolific . . . fatness and flabbiness are to blame. The womb is unable to receive the semen and they menstruate infrequently and little’ (Lloyd, 1978). While a women’s body fat is a vital prerequisite for reproductive efficiency and pregnancy, an extreme increase appears to lead to poor reproductive outcomes such as infertility, increased risk of miscarriage and a poor response to ART (Lewis et al., 1990).

1.2.1.1 Effects on sexual dysfunction

There is an increasing evidence to suggest that obesity not only affects physical health, but also has an adverse impact on sexual health. Sexual dysfunction is found to be more common in obese women when compared to slim women (Shah, 2009). The frequency of penile-vaginal intercourse is greater in slimmer women as measured by smaller waist and/or hips (Brody, 2004). Obese women may suffer from lack of libido and sexual satisfaction, have difficulty with sexual performance and avoid sexual encounters (Kolotkin et al., 2006). This may be a result of amalgamation of factors to include physical, psychological, hormonal, physiological, emotional, reproduction and social. In general slimmer physique is considered to be physically more attractive and as they are at a lower risk of metabolic, cardiovascular and malignant disorders, the potential partners react similarly, especially with regards to sexual behaviour (Brody, 2004). Obese women have difficulty in commencing sexual relationship because of low self-confidence about their physique and the associated symptoms of hyperandrogenism such as acne and hirsuitism. Associated medical conditions such as musculoskeletal disorders, urinary incontinence and menstrual disorders may restrict sexual function in obese women. Women with high BMI have lesser indices of brain dopamine activity (Doknic et al., 2002) and the association of overeating with increased serotonin levels in the brain (Hussanain and Levin, 2002) may result in poor sexual desire and increase sexual dysfunction.

1.2.1.2 Effects on fecundability

Many studies have reported an association between obesity and reduced fecundability or the probability of conceiving a pregnancy for a given cycle (Bolumar et al., 2000; Rich-Edwards et al., 2002; Hassan and Killick, 2004; Gesink Law et al., 2007; Yilmaz et al., 2009). It is possible that increased oestrogen levels as a result of increased peripheral conversion of androgen to oestrogen in adipose tissue, decreased availability of gonadotropin releasing hormone could impede with hypothalamic-pituitary-ovarian control of ovarian function resulting in anovulatory cycles (Pralong et al., 2002; Haslam and James, 2005).

Impaired fertility has also been established in women with regular menstrual cycles. A retrospective data (Gesink Law et al., 2007) demonstrated a reduced fecundity for overweight (OR 0.92, 95% CI 0.84 – 1.01) and obese women (OR 0.82, 95% CI 0.72-0.95). A large retrospective cohort study of 22,840 women demonstrated an association between obesity and reduced fecundity for all weight-adjusted groups of women (Yilmaz et al., 2009). Both these studies showed that the effect of reduced fecundity persisted even for women with regular cycles. The finding of reduced fecundity with regular menstrual cycle i.e. regular ovulation may be due to the possible effects of obesity on the endometrium or on the fertilization potential of the oocyte.

1.2.1.3 Effects on natural fertility

About 1 in 6 couple experience infertility. Most obese women are able to achieve pregnancy readily and anecdotally, many morbidly obese women do not have any difficulty achieving a pregnancy. Howe et al. (1985) suggested that height, weight or Quetelet's index did not show any significant association with fertility. A retrospective cross-sectional survey of 1081 women suggested that infertile women when compared to women without infertility had a 4.8 fold increase in being obese (OR = 2.02, 95% CI, 0.70 – 5.84) and 3.8 fold increased risk of being overweight (OR = 2.11, 95% CI, 0.72 – 6.07) (Esmaeilzadeh et al., 2013). A nested case control study (Nurses' Health study) analysed 2527 married nulliparous nurses with ovulatory infertility of at least 1 year and compared them to 46,718 married parous controls with no infertility. The multivariate relative risk of primary ovulatory infertility at the age of 18 was 1.3 fold higher (95% CI 1.2-1.6) for women with BMI of 24-31 and 2.7 fold more (95% CI 2.0-3.7) for women with a BMI of more than 32 (Rich-Edwards et al., 1994). The Nurses' Health study II by the same group suggested that ovulatory infertility was associated with overweight and sedentary lifestyle than underweight and overexertion (Rich-Edwards et al., 2002). It is likely that high BMI has a negative impact on natural fertility as a possible result of various factors.

1.2.1.4 Does weight loss improve fertility?

There is good evidence and a better understanding of the positive effects of weight reduction on improved reproductive outcome. A multi-centre, prospective, randomized, double blind, placebo controlled study assessed the effects of lifestyle measures plus placebo to lifestyle measures and metformin on changes in menstrual cycle, anthropometric measures and endocrine parameters in obese anovulatory PCOS women. The study elegantly showed that both groups of women showed a significant improvement in weight loss and increase in menstrual frequency (Tang et al., 2006). Norman et al. (2004) reviewed the available evidence on the effects of weight management and dietary intervention in improving female reproductive function and suggested that in obese women even a modest weight loss of about 10% could effectively improve ovulation, menstruation, pregnancy rates and hormonal profile. The review suggested that weight loss in PCOS women was found to improve insulin sensitivity, normalising androgen excess, decreased hyperlipidaemia and ovarian $p450_c17\alpha$ activity. Ferlitsch et al., (2004) analysed results of 171 women who underwent IVF or ICSI with particular reference to variations in BMI and day 3 FSH levels. They demonstrated that by increasing BMI by one unit, the odds for pregnancy decreased by 0.84 (95% CI 0.73-0.97). This suggests that chance of pregnancy increases with weight loss and any decrease in BMI by one unit improves pregnancy rates by 19%.

1.2.2 What are the effects of high BMI on Infertility treatment?

There is increasing evidence from the clinical studies that an increased BMI may be associated with poorer outcomes following treatment for infertility. NICE recommends that female BMI should ideally be in the range of 19-30 before considering assisted conception treatment, considering the likely reduction in treatment success for women outside this BMI range (NICE 2013). Many NHS trusts across the UK have female partner's BMI of ≤ 30 as one of the criteria for funding a couple's infertility treatment. The BFS guideline also recommends aiming for a normal BMI before considering any fertility treatment. It also advises clinicians to defer treatment until the BMI is less than 30 in women under the age 37 years and with normal ovarian reserve and that the treatment should not be offered to women with BMI ≥ 35 (Balen and Anderson, 2007).

1.2.2.1 Ovulation Induction

Clomiphene citrate remains one of the first line treatments for anovulatory infertility. Imani et al. (1998) demonstrated that in 201 normogonadotrophic, oligomenorrhoeic or amenorrhoeic infertile women who underwent ovulation induction (OI) with clomiphene citrate, 22.5% were clomiphene resistant and did not ovulate. The BMI was significantly more in the resistant group than in the responders and high BMI was found to be one of the predictors for women remaining anovulatory after clomiphene citrate OI.

Gonadotrophins are one of the second-line treatments for OI in clomiphene resistant anovulatory women. A meta-analysis that looked at 13 studies suggested that the total dosage of gonadotrophins required was more (WMD of 771 IU, 95% CI 700 – 842) in obese women with a significantly reduced ovulation rate (pooled OR 0.44, 95% CI 0.31 – 0.61) and an increased chance of cycle cancellation (pooled OR 1.86, 95% CI 1.13 – 3.06) (Mulders et al., 2003). The results of two large, multicentre OI studies that assessed the influence of obesity on OI outcome, suggested that obese women required a significantly higher dose of gonadotrophins and a longer duration of stimulation, and produced significantly fewer large follicles, but had no overall effect on ovulation and pregnancy rates (Balen et al., 2006).

1.2.2.2 Superovulation and in-utero insemination

A university-based, retrospective chart review of 333 ovulatory women who underwent superovulation and IUI for infertility treatment demonstrated that in obese women when compared to normal weight women, the adjusted cycle fecundity was no different, but the total gonadotrophin dose required was more and levels of oestradiol were less, but there was no difference in the number of larger follicles (Dodson et al., 2006). However, a retrospective study found that obesity was associated with a lower number of medium, large and total follicles for a given dosage of gonadotrophins, but no difference in the clinical pregnancy and miscarriage rates (Souter et al., 2011).

1.2.2.3 Assisted Reproduction

There is inconsistent evidence regarding the effects of obesity on ART outcome. Some studies have suggested that the BMI did not affect pregnancy rate after IVF treatment (Lashen et al., 1999; Matalliotakis et al., 2008), but the systematic reviews of observational studies, mainly retrospective, have demonstrated a possible harmful effect of obesity on pregnancy outcome (Maheshwari et al., 2007; Rittenberg et al., 2011a).

Pooling of results from five studies in a systematic review and meta-analysis that looked at IVF treatment outcomes reported a statistically significant decrease in the live birth rates in obese women and in the clinical pregnancy rates in overweight (pooled data from 16 studies) and obese women (pooled data from 15 studies) when compared to women with normal BMI (Rittenberg et al., 2011a). The same review also reported that obese women required more total dose of gonadotrophin and a longer duration of stimulation.

McLernon et al. (2010), in their meta-analysis evaluating the use of single embryo transfer on IVF outcome showed that for every unit increase in BMI, the odds of a very preterm delivery ≤ 32 weeks was increased by 16% (OR 1.16, 95% CI 1.04-1.30). Inherent bias is a common problem with pooling of observational studies data as it is difficult to adjust for confounding factors such as age, cause of infertility and the number and quality of embryos transferred.

A single largest study that looked at 31,672 ART embryo transfers from the Society of Assisted Reproductive Technology Clinic, which permitted adjustment for age, parity, number of embryos transferred and the embryo transfer day, showed that failure to achieve a clinical intrauterine gestation was significantly more likely for obese women (RR:1.22, 95%CI: 1.13-1.32) (Luke et al., 2011). However, a recent systematic review (Koning et al., 2012) that included 27 studies found no significant decrease in the rates of clinical pregnancy, ongoing pregnancy and live birth following ART in overweight and obese women when compared to normal weight women and the authors conclude that ART should not be restricted based on BMI.

There is a lack of good quality evidence to highlight the effects of woman's BMI on ART success and on the risk of complications. There is a real need for future well-designed prospective studies which should take into consideration co-morbidities and other factors, to advice clinicians of the likely effects of high BMI on ART outcome.

1.2.3 Obesity and Miscarriage

Spontaneous miscarriage affects 12-15% of all pregnancies (Zinman et al., 1996) and it is estimated that the risk of miscarriage in obese women may be as high as 25-37% before a first successful pregnancy (Hamilton-Fairley et al., 1992). Obesity is proposed to be an independent risk factor for early pregnancy

miscarriages (Fedorcsák et al., 2000). Most of these miscarriages occur in early pregnancy before 12 weeks of gestation.

Metwally et al., (2008), in their systematic review and meta-analysis of sixteen studies that investigated the association between obesity and miscarriage, showed that women with BMI of ≥ 25 had a significantly higher odds of miscarriage, irrespective of method of conception (OR 1.67, 95% CI 1.25-2.25).

The available evidence for miscarriage following spontaneous conception is based on a systematic review (Boots and Stephenson, 2011) that included three retrospective studies and one prospective study with a large cohort of women (n=24,738). The findings suggested that the risk of a miscarriage following spontaneous conception increased by 31% for obese women (OR 1.31, 95% CI 1.18-1.46) and 11% for overweight women (OR 1.11, 95% CI 1.00 – 1.24) when compared to women with normal BMI. None of the included studies in this systematic review had a sample size calculation and the pooled analysis did not account for maternal age and gestational age at miscarriage.

Pooled data from 22 observational studies, mainly retrospective, in a systematic review (Rittenberg et al., 2011a) that looked at ART treatment outcomes demonstrated a significantly increased risk of miscarriage in women with BMI ≥ 25 when compared to normal BMI women (RR 1.31, 95% CI 1.18 – 1.45, $p<0.00001$, $I^2 = 47.9\%$). There was no reported significant heterogeneity in the

included studies. Two studies in this systematic review were prospective with the rest retrospective and only one study was case-control whereas the rest were cohort studies. There was no adjustment for important confounders such as maternal age and the number and quality of embryos transferred, all of which are important predictors of ART success.

Rittenberg et al., (2011b) demonstrated that women with BMI ≥ 25 had more than double the risk of miscarriage when compared to normal BMI women in fresh and cryo thawed single blastocyst transfer ART cycles. When confounding variables such as female age, duration and cause of infertility, previous miscarriage, smoking status and quality of blastocyst replaced were adjusted, the odds of miscarriage was significantly higher for women with BMI ≥ 25 in fresh (OR 2.7, 95% CI 1.5-4.9) and cryo-thawed (OR 6.8, 95% CI 1.5-31.1) ART cycles and concluded high BMI as an independent risk factor.

There is much debate on the possible association between female obesity and miscarriage and most of the evidence comes from retrospective studies and systematic reviews of these studies which suggest of a possible increased risk of miscarriage both in spontaneous conceptions and pregnancies following ART. Maternal age has a significant effect on the miscarriage which is not controlled in most of these studies. There is an urgent need for well-designed prospective studies that can provide appropriate evidence on the effects of BMI on miscarriage and in understanding the underlying mechanisms, which will be invaluable in counselling women about these risks.

1.2.4 Obesity and Recurrent Miscarriage

The loss of three or more consecutive miscarriages before 24 weeks of gestation is traditionally termed as recurrent miscarriage (RM) and it affects 1-2% of the couple trying to achieve a pregnancy (Stirrat GM, 1990). In about 50% of all cases, the cause remains unknown despite a wide range of investigations (idiopathic RM) and in the remaining, the known aetiological factors include genetic, thrombophilic, endocrine, immune, infection and anatomical or structural causes (RCOG green-top guideline, 2011).

Two retrospective studies (Lashen et al., 2004; Metwally et al., 2010) have analysed the association between obesity and RM. A matched case control study by Lashen et al. (2004) that included 1644 obese and 3288 normal weight women with a mean age of 26.6 years showed a significantly higher odds of RM in obese women (OR 3.51, 95% CI 1.03 – 12.01). Metwally et al., (2010) looked into a total of 844 pregnancies from 491 women with RM and found out that obese women, when compared to women with normal BMI had a significantly increased odds of miscarriage in a subsequent pregnancy (OR 1.71, 95% CI 1.05 – 2.8). Using logistic regression analysis, they analysed the data further to conclude that advanced maternal age was the most important predictor of miscarriage followed by increased maternal BMI.

1.3 Metabolic effects of obesity on reproduction

From the available clinical studies, it is evident that the obesogenic environment has an adverse effect on reproductive outcomes, but the mechanism by which obesity causes increased reproductive failures is not fully understood. It is possible that the metabolic disturbances associated with obesity may exert effects on the ovary, the embryo and endometrium. The obese environment may also cause perturbations in hormonal balance that control ovulation and endometrial development.

1.3.1 Effects on ovary

1.3.1.1 Polycystic ovarian syndrome (PCOS)

PCOS is a common endocrine disorder in women of reproductive years with a prevalence as high as 15% (Fauser et al., 2012), and is responsible for 90-95% of women with anovulatory infertility attending infertility clinics (Balen and Anderson, 2007). Obesity is common in women with PCOS and it is estimated that at least 35-40% of women with PCOS are obese (Hamilton-Fairlley et al., 1992; Balen et al., 1995). Some studies have reported a prevalence of obesity to be as high as 61-76% in American and Australian PCOS women (Ching et al., 2007; Glueck et al., 2005). Though obesity is closely linked to PCOS, the

mechanism to understand the evolution of obesity and PCOS are not fully understood (Fauser et al., 2012).

The Rotterdam consensus workshop (2004) set out diagnostic criteria to diagnose PCOS which included cardinal features of ovulatory dysfunction hyperandrogenism and polycystic ovary morphology. Ovulation dysfunction is characterised by chronic oligo or anovulation represented by oligomenorrhoea or amenorrhoea. In a cohort of Srilankan women, 95% of PCOS women were found to have oligo- or amenorrhea (Kumarapeli et al., 2008). Ovulation in PCOS women may occur spontaneously in up to 32% of the cycles, but the frequency of ovulation is unknown (Laven et al., 2002). The extent of ovulatory dysfunction, androgen levels and follicular count depends on whether a PCOS woman has oligo-ovulation or anovulation (Burgers et al., 2010).

Hyperandrogenism in women clinically presents as hirsuitism, acne and androgenic alopecia, and are present in about 74%, 53% and 34.8% respectively of women with PCOS (Ozdemir et al., 2010). Hyperandrogenaemia or excessive circulating free androgen levels may either be due to excessive production from the ovary or adrenal glands or by a decreased SHBG synthesis in liver. A proportion of PCOS women may not exhibit hyperandrogenaemia (Pugeat et al., 1993; Balen et al., 1995; Laven et al., 2002). Many PCOS women have insulin resistance (IR), defined as decreased insulin-mediated glucose utilisation with compensatory hyperinsulinaemia (Dewailly, 1997). In women with PCOS, the degree of metabolic dysfunction correlates strongly with

the amount of visceral fat, which either causes or pre-disposes a woman to IR (Lord et al., 2006). Increased visceral adiposity is associated with greater IR, which could exacerbate the abnormalities in reproductive function of women with PCOS (Balen and Anderson, 2007). Although IR can occur irrespective of BMI, the association of PCOS and obesity have a synergistic adverse impact on glucose homeostasis and worsen both hyperandrogenism and anovulation. The hyperandrogenism and hyperinsulinaemia along with other extra and intra-ovarian factors in PCOS may adversely affect oocyte and embryo quality (Qiao and Feng, 2011). Hyperinsulinaemia is found to be an independent risk factor for first trimester miscarriages with an OR of 1.32 (95% CI 1.09-1.60) for every 5 µIU/ml increment in insulin levels (Glueck et al., 2002).

1.3.1.2 Ovarian dysfunction

It is proposed that obesity may cause an adverse effect on the ovarian function either by its metabolic effects on ovulation or on the oocyte quality. In ART cycles, controlled ovarian hyperstimulation (COS) is undertaken to obtain sufficient number of oocytes and it is suggested that the ovaries in obese women may be more resistant to COS. However, two systematic reviews (Maheshwari et al., 2007; Ritteberg et al., 2011) have pooled the results of mainly retrospective observational studies that looked into the effects of overweight and obesity on ART cycles and both concluded that there was no difference in the number of oocytes recovered in different BMI groups. The most recent systematic review and meta-analysis pooled the results from five studies

that compared the duration and total dosage of gonadotrophins used for COS in different weight groups. The review reported that obese women required a higher dose of gonadotrophin stimulation (WMD 406.77, 95% CI 169.26-644.2, p=0.0008) and a longer duration of stimulation (WMD 0.27, 95% CI 0.26-0.28, p<0.00001) when compared to women of normal BMI. Though there was a significant heterogeneity between the included studies that reported the dose of gonadotrophins used (I^2 = 80.8%), there was no significant heterogeneity between the included studies for the duration of gonadotrophins used (I^2 = 0%) (Rittenberg et al., 2011). The risk of ART cycle cancellation (pooled data from 3 studies out of which two were due to poor response) was higher in women with BMI>30 with an OR 1.34 (95% CI 0.99-1.84) when compared to women with BMI <30. This suggests that obese women when compared to normal weight women, to produce similar number of oocytes in response to COS in ART cycles' require longer duration of gonadotrophins stimulation and possible more gonadotrophins. It is well recognised that drug bioavailability is decreased and drug distribution is altered in obese women, which may explain these findings.

The evidence on the effects of obesity on oocyte quality is conflicting. It has been accepted that on a morphological assessment, an oocyte with a clear to colourless zona pellucida, clear or moderately granular cytoplasm and a small perivitelline space is considered a good quality metaphase II oocyte (Veeck, 1988). Wittemer et al. (2000) morphologically analysed the oocytes obtained from women undergoing IVF or ICSI (332 cycles with long protocol, 50 cycles with short protocol and 16 cycles without gonadotrophins) and showed that

women with BMI ≥ 25 had significantly poor quality oocytes retrieved when compared to the oocytes obtained from women with BMI <25.

During IVF, hCG is given in a higher dose (5,000-10,000IU) to imitate mid-cycle LH surge and hcg when present in adequate levels in the stimulated follicles, helps in the completion of the first meiotic division of an oocyte to become a MII oocyte and ready to undergo fertilization. Using this principle, Carrell et al., (2001) prospectively assessed follicular fluid hCG concentrations from 247 consecutive IVF cycles and demonstrated a significant inverse correlation between intra-follicular hCG concentration and BMI. They also found significantly reduced mean hCG concentration, poor embryo quality and decreased clinical pregnancy rates in women with BMI ≥ 30 when compared to women with BMI 20-30, but no difference in oocyte quality.

The principal mechanisms that govern the appearance of an oocyte are multi-factorial and complex and make the morphological assessment of an oocyte a difficult process (Balaban and Urman, 2006). Hence, fertilization rate in ART cycles is considered a surrogate marker and a more objective way of predicting oocyte quality. Fedorcsak et al., (2004) in their large cohort of 5019 IVF or ICSI cycles did not find a significant correlation of BMI to diploid fertilization rates. Dokras et al., (2006) demonstrated that there was no significant difference in the fertilization rates, the number of embryos transferred and cycle cancellation as a result of failed fertilization in different BMI groups. A retrospective analysis of 426 IVF/ICSI cycles that assessed the fertilization rates between different

BMI groups suggested that obesity did not have any significant effect on oocyte quality (Metwally et al., 2007). Esinler et al. (2008) looked at the impact of isolated obesity on the outcome of ART in a total of 775 patients undergoing 1113 ICSI cycles. They reported reduced number of cumulus-oocyte complex, MII oocytes and oocytes with 2 pro nuclei in obese women compared to women with normal BMI, but did not find any significant difference in fertilization rates. A large retrospective analysis of 6500 IVF cycles failed to establish an association between BMI and the number and maturation of oocytes and fertilization rates, but clearly showed a reduction in implantation, pregnancy and live birth rates (Bellver et al., 2010). Another retrospective cohort study of 1,721 women undergoing a first IVF cycle with fresh, autologous embryos between 2007 and 2010 concluded that the number of oocytes at metaphase II stage and the failed fertilization rates were comparable in different BMI groups (Shah et al., 2011).

In conclusion, negative environmental influence may affect the developmental competence of the oocyte and the available evidence suggests that obesity when associated with PCOS may affect ovarian function by altering regular ovulation. The systematic reviews of the studies have produced conflicting results. The number of oocytes produced by obese women in response to COS in ART cycles is found to be similar to normal weight women suggesting that the metabolic effects of obesity may not be mediated through ovarian function. However, it is essential to recognise that the evidence comes mainly from retrospective cohort studies. Also, there is no good quality evidence from morphological assessment of oocytes and fertilisation rate as a surrogate marker, to conclude that obesity has negative influence on oocyte quality.

1.3.2 Effects of obesity on an embryo

Successful implantation of an embryo into the endometrium is an intricate procedure that depends on the synchronized cross-talk between a developmentally competent embryo and a receptive endometrium (Dey et al., 2004). Chromosomal abnormalities are common in pre-implantation embryos. Any alteration during meiosis would result in abnormality in all the cells whereas any error during the first mitotic division is responsible for chromosomal mosaicism (Koot et al., 2012). A study by Munné et al., (2007) looked at more than 6000 embryos of which many were suitable for embryo transfer. They found that 44% of the embryos with best morphology and development were euploid in women under the age 35 and only 21% in women aged 41 or more. Vanneste et al. (2009) examined the incidence of chromosomal abnormalities by comparative genomic hybridization screening in cleavage stage IVF embryos obtained from young fertile women under the age of 35 years. Only two out of 23 good quality embryos were found to have normal karyotype in all blastomeres. Nearly fifty percent of the embryos had karyotypically no normal cells and the remaining embryos were mosaic for structural chromosomal imbalances. They proposed that the prevalence of chromosomal abnormalities in the pre-implantation embryos may be as high as 90%. Karyotype of products of conception after three or more consecutive pregnancy losses indicate that aneuploidy is the most common cause of RMC (Sugiura-Ogasawara et al., 2010). An exceptional feature of implantation is to overcome such a high prevalence of chromosomally abnormal embryos. Most of the aneuploid

pregnancies (90%) miscarry whereas 93% of karyotypically normal pregnancies continue – natural embryo selection (McFadden, 1989).

The available clinical evidence indicating the consequences of high BMI on embryo quality is inconsistent and debatable (Carrell et al., 2001; Fedorcsak et al., 2004; Metwally et al., 2007; Esinler et al., 2008; Zhang et al., 2010; Bellver et al., 2010; Zander-Fox et al., 2012). Carrell et al., (2001) observed that women with BMI ≥ 30 had significantly poorer quality embryos than in women with BMI 20-30. Some authors (Metwally et al., 2007; Esinler et al., 2008) have considered overall quality of the embryos produced in an ART cycle and not just the embryo grading to assess the overall quality. Metwally et al. (2007) assessed four different markers for embryo quality (embryo grade, embryo utilization rate, number of embryos discarded and the number of embryos cryopreserved) in a retrospective analysis of 426 consecutive IVF cycles and reported significantly poor quality embryos in obese women under the age of 35 years. Esinler et al. (2008) found a significantly less number of surplus embryos cryopreserved in obese when compared to normal BMI women (10.7% vs.22.7%). Zhang et al. (2010) in their retrospective cohort study of 2628 women in China suggested that obese women have lower fertilisation rates, fewer cleaved and high grade embryos and fewer embryos for cryopreservation.

However, a large retrospective study on 6500 IVF/ICSI cycles concluded that the embryo quality was not impaired in overweight and obese women and suggested of a possible endometrial cause for poorer IVF outcomes in obese

women (Bellver et al., 2010). There was no association between obesity and morphologic characteristics of the embryo found in another retrospective cohort study looking at 1721 women undergoing first cycle of fresh IVF treatment suggesting that obesity may exert an effect on reproduction without affecting embryo quality (Shah et al., 2011). In another retrospective study of IVF cycles, BMI did not have an apparent effect the embryo development in women less than 38 years of age (Zander-Fox et al., 2012).

In summary, there is again a paucity of good quality evidence to suggest that obesity impacts developmental competence of an embryo. Active research need to be undertaken to determine complex interactions of various hormones and metabolites in obesity may affect oocyte and embryo quality. Also, there is also an urgent need for well-designed studies to assess the effects of weight loss on ovarian function as it appears that the intervention may ameliorate the negative effects of obesity on reproduction.

1.3.3 Effects of obesity on endometrium

Endometrium plays a major role and is likely to be the most influential factor in implantation. Cyclic decidualisation of the human endometrium prepares the uterus for implantation of a developmentally competent embryo, to identify and reject developmentally abnormal embryos and also prepares the endometrium for deep placentation in pregnancy (Teklenburg et al., 2010a). Bellver et al.

(2007) suggested that the ovum donation model (that looked at ART cycle outcome in oocyte recipients and the oocytes were obtained from healthy donors) as an acceptable human model for discriminating the effects of obesity on endometrium from its effects on the ovary. Few studies have endeavoured to outline the effects of obesity on the endometrium (Wattanakumtornkul et al., 2003; Styne-Gross et al., 2005; Bellver et al., 2003, 2007, 2013)

A small study from the University of Minnesota looking at 97 women having their first cycle of IVF using donor oocytes concluded that uterine receptivity was not altered in obese women when hormonal support and embryo quality were standardised (Wattanakumtornkul et al., 2003). Bellver et al., (2003), in their first study looking at 712 cycles of ART using donor oocytes found that the miscarriage rate was fourfold (OR 4.02, 95% CI 1.53-10.57) in obese women when compared to women with normal BMI and concluded that obesity is an independent risk factor for miscarriage in women receiving donor oocytes.

Styne-Gross et al., (2005) retrospectively looked at data of 536 donor oocyte recipients. The donors were healthy females with an average age of 26.8 years and an average BMI of 22.2 +/- 2.3. There was no significant difference in implantation rates, on-going pregnancy rates and miscarriage rates in obese women when compared to women with normal BMI. The ROC curves failed to demonstrate BMI as a risk indicator for spontaneous miscarriages.

Bellver et al., (2007) investigated 2656 women undergoing first egg donation cycles suggested that the on-going pregnancy rate was significantly lower in overweight and obese women compared to women with normal BMI and linear regression analysis demonstrated a negative trend in pregnancy rates with increasing BMI. They concluded that endometrial receptivity was linked to the detrimental effects of obesity. The biggest study so far by Bellver et al. (2013) which looked at the outcome of 9587 first cycle of egg donation recipients showed that implantation, clinical pregnancy and live birth rates were significantly reduced in the obese group with no difference in miscarriage rates.

It was argued that co-morbid conditions in the recipients may be contributing to the adverse reproductive outcomes in oocyte donation cycles. To overcome this factor, DeUgarte et al. (2010) designed a third party assisted reproduction models of IVF which was a retrospective study using healthy donor oocytes (n=551) and healthy surrogates (n=341) (except for the BMI). A significant decrease in live birth rates was observed in women with BMI >35 when compared to women with BMI <35. This observation reinforces the other findings that impaired endometrial receptivity in obese women is the main responsible factor for poor reproductive outcomes.

A retrospective case-control study that looked at data of embryonic karyotype of products of conception after surgical evacuation of first trimester miscarriages in women less than 40 years of age (n=204), suggested that overweight/obese women had an increased rates of miscarriage of euploid embryos (Landres et

al., 2010). They concluded endocrine disorders and altered endometrial milieu may contribute to these miscarriages. It is plausible that the obesogenic environment impairs the peri-implantation endometrium, which may create an unfavourable milieu for successful implantation even when embryos are karyotypically normal.

In summary, it appears from these studies that an obesogenic environment may have a negative influence on the reproductive health of a woman. It is possible that an obese woman may take more time to conceive, may have more miscarriages, following both spontaneous and assisted conception and may have reduced clinical pregnancy and live birth rates following assisted conception treatment. Various studies have been undertaken to assess the direct, metabolic and endocrinological influence of obesity on reproduction and it is interesting to note that the obesogenic effects are likely to be on the endometrium and less likely to be on the ovarian function, unless associated with PCOS, and on the embryo quality. The exact mechanism by which the obesogenic environment affects the peri-implantation endometrial function remains to be fully clarified.

1.4 Human endometrium

The human endometrium is a multi-layered and an extremely dynamic tissue. It cyclically proliferates, differentiates, sheds and then regenerates in response to ovarian steroids approximately 400 times in a woman's reproductive lifespan (Jabbour et al., 2006; Brosens and Gellersen, 2006). The endometrium has two main components, the stratum functionalis and stratum basalis. The superficial functionalis extends from the surface epithelium to endometrial/myometrial junction and sheds at each menstruation. The deep stratum basalis is attached to the myometrium and encompasses the basal region of endometrial glands, dense stroma and large vessels serving as a base for endometrial regeneration. The human endometrium is characterised by surface epithelium, cellular stroma with stromal fibroblasts, glands, immune cells and blood vessels. The cellular composition, structure and function of the endometrium vary throughout the menstrual cycle which is divided into three phases – the proliferative, the secretory and menstrual. Important changes in phenotype and/or abundance of most of these cells occur mainly in the peri-implantation period (Gellersen et al., 2007) which at functional level are defined as the fertile window, the implantation window and the window of natural embryo selection (Teklenburg et al., 2010).

1.4.1 The phases of endometrial changes in a menstrual cycle

1.4.1.1 The proliferative phase

The proliferative phase is the initial phase of the menstrual cycle that starts immediately after menstruation and lasts until ovulation. During the proliferative phase of a menstrual cycle, the dominant hormone is ovarian oestradiol which encourages structured growth and proliferation of the endometrium. A reduction in circulatory oestrogen levels during the last few days of the previous menstrual cycle prompts a negative feedback on the hypothalamus to increase the secretion of gonadotrophin releasing hormone (GnRH), which in turn increases the production and release of follicular stimulating hormone (FSH) from the anterior pituitary gland. FSH induces follicular development and maturation in the ovaries, in which several antral follicles are recruited to develop one mature dominant follicle (Graafian follicle). FSH is responsible for proliferation of granulosa cells in the developing follicles, which are the main source of oestrogen production. The androgens that are produced in the theca cells diffuse into the granulosa cells to be aromatised under the influence of FSH to oestrogens – predominantly oestradiol and to a lesser extent oestrone. Theca cells and the ovarian stroma also have a small contribution to the production of oestrogen.

The proliferative changes in the endometrium occur as a result of increasing ovarian oestrogen levels. The short endometrial glands elongate and are arranged perpendicular to the surface. The epithelium acquires columnar features with the nuclei placed at the base. The stromal cells become spindle shaped and the spiral vessels form a loose capillary network in the sub-epithelial region. The adult endometrial mesenchymal stem cells are undifferentiated cells present in the basal layer of the human endometrium and are hypothesized to be responsible for the cyclical regeneration of the functionalis after menstruation (Gargett, 2007). Under the influence of oestrogen, several growth factors such as Epidermal growth factor (EGF), transforming growth factor (TGF)-alpha, platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and their binding proteins, fibroblast growth factor (FGF), TGF-beta, colony-stimulating factor (CSF)-1 and vascular endothelial growth factor (VEGF) regulate the proliferation of the endometrium (Giudice, 1994; Chan et al., 2004).

1.4.1.2 The secretory phase:

The secretory phase is the next phase of the menstrual cycle that begins with ovulation and ceases a few days before menstruation. A rise in the levels of oestrogen towards the end of the follicular phase triggers a surge in levels of luteinising hormone (LH) and FSH secretion from the anterior pituitary gland. This spike in levels of LH results in the rupture of the dominant follicle inducing

ovulation. The follicle in the ovary transforms to corpus luteum which secretes progesterone.

The changes in the endometrium occur due to the combined effects of oestrogen and progesterone and the progesterone can only act on the endometrium primed by oestrogen. The surface epithelial cells become more columnar and in some places acquire cilia. The glands increase in size and show predominant changes. Sub-nuclear vacuolation occurs in the glandular epithelial cells (Noyes et al., 1950) in which the vacuoles appear between the nuclei and the basement membrane due to accumulation of glycogen. The glycogen rich intra-cellular secretion is released into the glandular lumen, which then drains into the uterine cavity. The stromal cells undergo morphological and biochemical transformation from elongated, spindle-shaped stromal fibroblasts into rounded, secretory, epitheloid like decidual cells (Gellersen and Brosens, 2003). Vascular remodelling occurs that is characterised by development and coiling of spiral arteries and growth of subepithelial capillary plexus (Gambino et al., 2002). There is a massive infiltration by immune cells mainly the uterine natural killer cells (uNK cells) and to a lesser extent macrophages and T cells (Bulmer et al., 1991a).

1.4.1.3 The menstrual phase:

In the absence of a pregnancy, the ovarian hormone levels decrease as a result of the degenerating corpus luteum. The decline in ovarian steroids induces a series of vascular, molecular and cellular changes by expression of cytokines, chemokines and matrix metalloproteinases and cause shredding of the superficial layer of endometrium which results in menstruation (Jabbour et al., 2006; Brosens and Gellersen, 2006).

1.4.2 The functional periods of menstrual cycle

1.4.2.1 The fertile window

The probability of achieving a pregnancy is highest with intercourse occurring near the time of ovulation. A prospective cohort study by Wilcox et al., (1995) analysed 625 ovulatory cycles in 217 women who were planning to achieve a pregnancy. They suggested that the conception can only occur with sexual intercourse on or during five days prior to ovulation. This period of six days just prior to ovulation and ending on the day of ovulation is termed as the'fertile window' (Wilcox et al., 1995). However it is possible that there is a variation in the length of the fertile window between couples and in different menstrual cycles. A prospective study that looked at the fertile window among subfertile

couples, based on sperm-mucous interaction, concluded that the fertile window is variable from <1 day to >5 days (Keulers et al., 2007). Based on the above, it would be tempting to assume that an extended fertile window would increase fertilization and conception rates, but in normal couples, fertilization does not appear to be the rate-determining step (Teklenburg et al., 2010 a).

1.4.2.2 The implantation window

The post-ovulatory rise in progesterone mediates changes within the endometrium and prepares it to respond to embryonic signals. This transient period of endometrial receptivity is termed as the 'implantation window', which starts around six days after ovulation and lasts for about four to five days (Macklon et al., 2002). This restricted duration of implantation window may be responsible to orchestrate embryonic development with that of the endometrial preparation (Wilcox et al., 1999) and also decreases the possibility of a late implantation of poor quality embryos. Further restriction of this crucial implantation window causes subfertility and IVF treatment failures (Boomsma *et al*., 2009; Devroey *et al*., 2009). Late implantation of the embryos is associated with an increased risk of early miscarriage (Wilcox et al., 1999). Hence it is possible that prolonged expression of endometrial receptivity carries the risk of allowing developmentally slow or poor quality embryos to implant thus increasing implantation rates, but presenting as early pregnancy losses. The signals that end the implantation window make sure that only those embryos

that have achieved developmental competency are implanted into the endometrium within this restricted period.

1.4.2.3 The window of natural embryo selection

The end of implantation window coincides with decidualisation of the stromal compartment which occurs in the mid-late luteal phase of the menstrual cycle independently of pregnancy This functional window in which decidualising endometrial stromal cells facilitate maternal identification and rejection of developmentally compromised embryos is named as 'the window of natural embryo selection' (Teklenburg et al., 2010). Decidualisation also prepares the endometrium for deep placentation in pregnancy. Any abnormality in the window of natural embryo selection weakens the embryo biosensor quality predisposing to miscarriage and recurrent miscarriages. Placental formation is also impaired causing obstetric complications such as pre-eclampsia, intra-uterine growth restriction and preterm delivery (Teklenburg et al., 2010).

1.4.3 Decidualisation of endometrial stromal compartment

The word 'decidua' is derived from the Latin '*decidere*' that means 'to fall off'. The increase in progesterone levels in the luteal phase of the menstrual cycle allows extensive modifications in the endometrium by secretory transformation of the endometrial glands, and by facilitating the differentiation of stromal cells

that involves morphological and biochemical transformation of elongated, spindle-shaped stromal fibroblasts into rounded, secretory, epitheloid like decidual cells that acquire myofibroblastic function (Gellersen and Brosens, 2003). It inhibits the proliferation of endometrial epithelium and generates vascular remodelling characterised by development and coiling of spiral arteries and growth of subepithelial capillary plexus (Gambino et al., 2002). Influx of immune cells occurs mainly by the uterine natural killer cells (uNK cells) and to some effect of macrophages and T cells (Bulmer et al., 1991). The stromal cells increase in size by at least five times and their identification based on their morphology becomes easy with their peculiar appearance of pale staining cytoplasm with an open vesicular nucleus on staining with haematoxylin and eosin (King, 2000). This well-designed remodelling of the endometrium allows it to transiently acquire a receptive phenotype. The initiation of decidualisation of the stromal compartment occurs in response to elevated progesterone and increasing levels of cellular cyclic-AMP (Gellersen and Brosens, 2003), and a continuous progesterone signalling is required for the maintenance of a receptive phenotype. Successful implantation guarantees persistently raised progesterone levels, which ensure decidual survival and integrity during trophoblast invasion and placenta formation (Brosens and Gellersen, 2006).

The process of differentiation originates in the stromal cells that surround the terminal portion of the spiral arteries (Streeter's columns) during the mid-secretory phase of the cycle (de Ziegler *et al*., 1998). Decidualisation eventually spreads to encompass the entire superficial endometrial layer by the late luteal phase of the cycle (Teklenburg *et al*., 2010), and in pregnancy it spreads to the

basal layer which is crucial for trophoblast invasion, and in formation of the placenta (Brosens et al., 2002). The stromal cell differentiation induces expression of several proteins, cytokines, growth factors and neuropeptides that promote endometrial receptivity. The forkhead box protein (FOXO1) is significantly induced upon decidualisation and is a key regulator of decidualisation (Gellersen and Brosens 2003). FOXO1 is instrumental for the expression of various phenotypic markers that include prolactin (PRL), insulin-like growth factor binding protein – 1 (IGFBP-1), left-right determination factor 2 (LEFTY2), wingless-type MMTV integration site family, member 4 (WNT4) and WNT16 (Takano et al., 2007). The first two are commonly used as phenotypic markers of decidualisation.

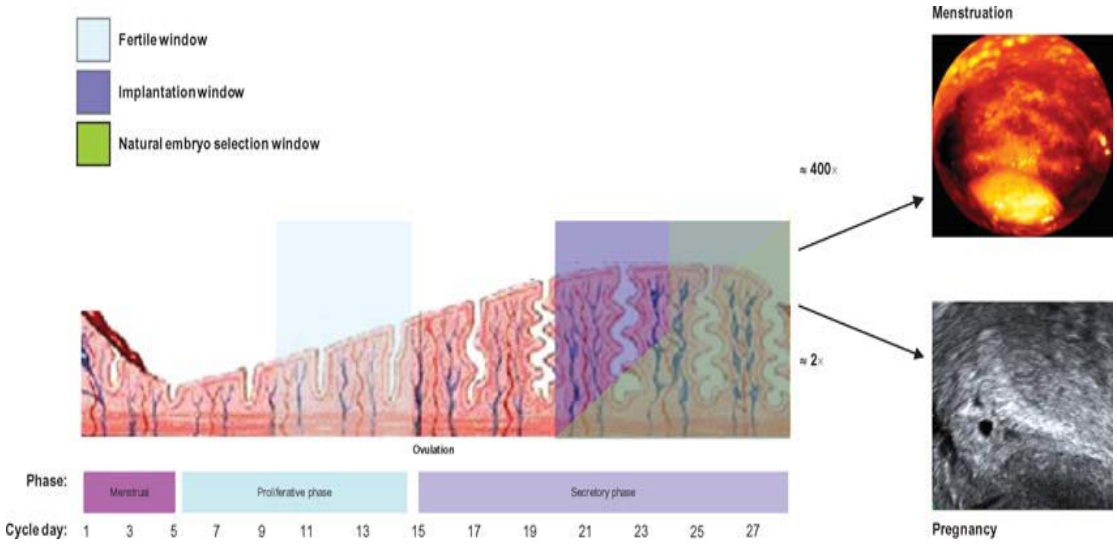


Figure 1.1: The functional windows of the menstrual cycle essential for reproduction. The likelihood of conception is determined by highly coordinated changes in the cycling endometrium, which at a functional level are defined as the fertile window, the implantation window and the functional window of natural embryo selection. Every cycle either leads to menstruation (≈ 400 times in a woman's lifetime) or to pregnancy (≈ 2 times in a woman's lifetime). (with permission from Oxford Journals: Teklenburg et al., (2010) The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod. 16(12):886-95.)

1.4.3.1 Decidualisation: Endometrial receptivity and Biosensors of embryo quality

The process of decidualisation in humans, which is initiated in the mid to late luteal phase of the menstrual cycle, is regulated under maternal control regardless of a pregnancy or implantation (Teklenburg et al., 2010a). The spontaneous, cyclical decidual transformation is essential for humans which enable the endometrium to transiently achieve a receptive phenotype. The endometrial response to individual embryos varies and depends on embryo quality (Figure 1.2). The decidualising endometrial stromal cells (ESC) acquire an incredible competence to reply to individual embryonic signals upon implantation and to function as 'bio-sensors' (Teklenburg 2010b), which serves as a mechanism for maternal recognition, prompt rejection and disposal of compromised embryos.

The in-vitro studies undertaken by Teklenburg et al. (2010b) to recognize the vital factors of implantation used a human co-culture model of decidualising stromal cells and hatched blastocysts. They elegantly demonstrated that out of the 14 secreted cytokines, chemokines and growth factors associated with implantation, the arresting embryo triggered a strong inhibitory response to eight factors. This suggests that the decidualising stromal cells react to a morphologically poor quality embryo by closing down the secretion of key implantation factors. However, a developmentally competent embryo caused no significant alteration in the secretions of implantation mediators from the

decidualising ESCs, except for a modest reduction in the expression of interleukin-5 levels. The blastocysts, irrespective of embryo quality, made no difference to the secretion of implantation factors when co-cultured with undifferentiated ESCs.

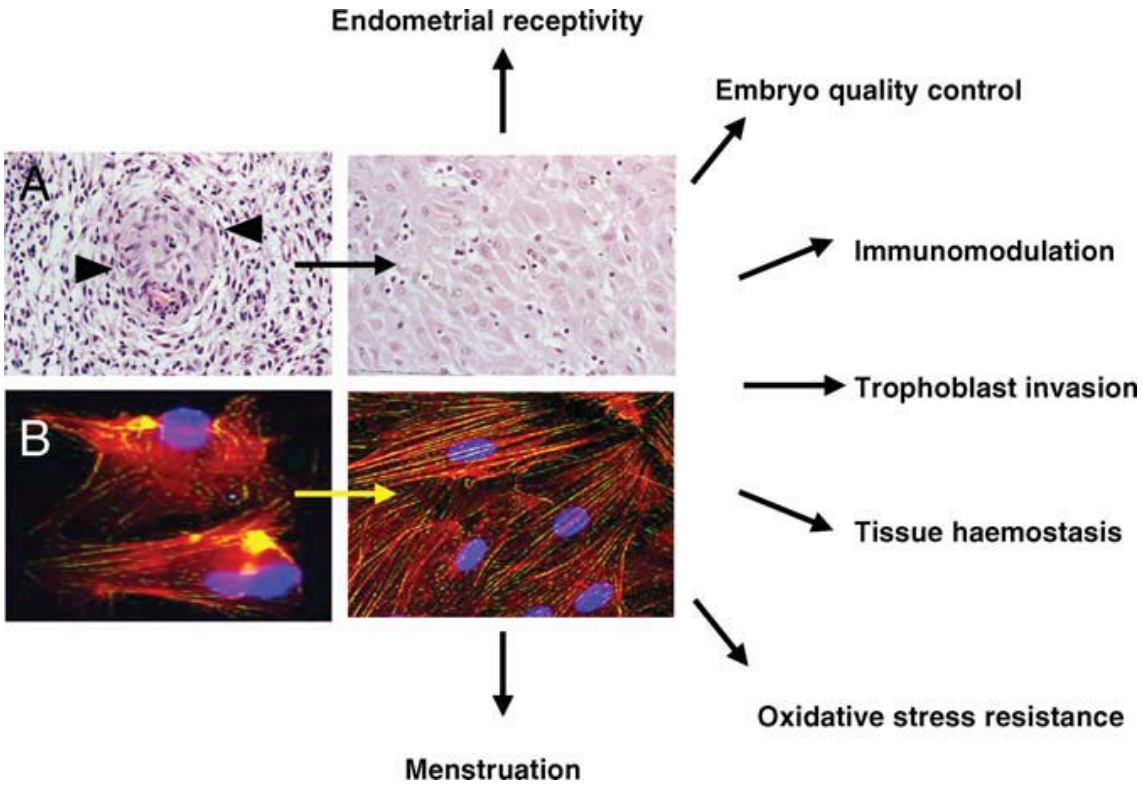


Figure 1.2: Decidual transformation of human endometrial stromal cells in vivo and in vitro. (A) Decidualization of the stromal compartment (arrow-heads) is initiated during the mid-secretory phase of the cycle in stromal cells that surround the terminal portion of the spiral arteries (left panel) and then spreads to encompass the entire superficial endometrial layer by the late luteal phase of the cycle (right panel). (B) Phalloidin staining of fila- mentous actin in undifferentiated and decidualized primary endometrial stromal cells in culture (left and right panels, respectively) highlights the phe- notypic reprogramming of the cells that underpins their very diverse functions. Decidualization is characterized by a dramatic increase in filamentous actin polymerization and stress fibre formation (red staining). The nuclei were stained with 4',6-diamidino-2-phenylindole (blue stain). (with permission from Oxford Journals: Teklenburg et al., (2010) The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod. 16(12):886-95.)

1.4.3.2 Impaired endometrial decidualisation and reproductive failures.

If decidualisation of the stromal compartment is critical for endometrial preparation for a successful pregnancy, it is acceptable to envisage that impaired decidualisation causes adverse pregnancy outcomes. The evidence from population based studies denotes that any perturbations in endometrial receptivity and natural embryo selection increase the risk of early pregnancy problems. A cohort study by Wilcox *et al*. (1999) examined from daily first morning urine samples from 221 women with no fertility problems, but trying to conceive, for markers of ovulation (estrone 3-glucuronide and pregnanediol 3-glucuronide) and hCG as a marker for implantation. They demonstrated that in successful pregnancies, implantation occurred 8 to 10 days post-ovulation, whereas a dramatic increase in early pregnancy losses was noted when the implantation occurred beyond the window of implantation. Jukic et al., (2011) found that late implantations were more likely to be associated with early pregnancy losses and to a lesser extent with surviving conceptions.

Biological evidence for endometrial dysfunction associated with early pregnancy failure originates from histological evaluation of the peri-implantation endometrium using traditional dating criteria (Noyes et al., 1950). Two morphological studies that followed strict criteria for comprehensively investigating for RMC and in obtaining a timed endometrial biopsy, reported that 17.4 - 27% of women with RMC were found to have delayed peri-implantation

endometrial development (Tulppala et al., 1991; Li et al., 2000). It was found that 60% of the biopsies from women with RMC had restricted endometrial development (Serle et al., 1994) when a morphometric analysis, a quantitative analysis of the changes involving different endometrial components, of the endometrium was undertaken. Studies have attempted to measure the concentrations of secretory proteins from the uterine flushing of women with RMC. These secretory proteins reflect the secretory activity of the endometrial glands and of the epithelium. Two important proteins, MUC1 and placental protein 14 (PP14) or glycodelin, are found to be reduced in women with RMC (Hey et al., 1994; Dalton et al., 1995; Salim et al., 2007).

The in-vitro experiments on decidualising ESCs provide further evidence on the impaired endometrial receptivity and poor reproductive outcome, particularly RM. Salker et al. (2010) measured the expression of two marker genes in the decidualising ESCs - Prokineticin-1 (PROK 1) and PRL. PROK1 is a vital cytokine which regulates endometrial receptivity via induction of leukaemia inhibitory factor in endometrial epithelial cells and PRL is a marker for decidualisation. They found that significantly higher levels of PROK-1 and markedly low levels of PRL were demonstrated in the decidualising ESCs from women with RM when compared to controls. These findings are suggestive of an intrinsic impairment of endometrial decidual transformation and uterine receptivity in women with RM.

Endometrial gene expression studies using microarray analysis of the endometrial biopsies obtained in the implantation window provide detailed information on endometrial receptivity. Many genes that are involved in distinct biological functions of cellular signalling (by inhibition and degradation of cyclic-AMP and abnormal calcium metabolism), cellular function and maintenance (lymphocyte homeostasis), nucleic acid metabolism and small molecule biochemistry are differentially expressed at the time of endometrial receptivity in women with RM. In RM, the humoral immune response and organ and muscle development are highly differentially regulated when compared to fertile controls (Lédée et al., 2011).

SGK1, a serine-threonine protein kinase, is induced by an increase in progesterone levels and executes an important role in cellular stress response. SGK1 is found initially in the luminal epithelial cells and then in the decidualising ESCs and plays a crucial role in early pregnancy (Feroze-Zaidi et al., 2007). Salker et al., (2011) found that the mid-secretory endometrial SGK1 transcript levels in women with RMC were markedly lower, when compared to fertile controls by day 4 of differentiation in cell cultures. They also demonstrated in immunohistochemical studies that phosphorylated SGK1, which reflects activated kinase, were lower in the stromal compartment of the endometrium of women with RM. In pregnant mice, they found that a lack of SGK1 in the decidua did not impair implantation, but was associated with bleeding and inflammation at the feto-maternal interface, and other events similar to obstetric complications such as miscarriage, growth restriction and stillbirth in humans.

Finally, further work by Salker at al. (2012) described that ESCs upon decidualisation mount a transient pro-inflammatory response and these signals activate the expression of endometrial epithelial receptivity genes followed by an anti-inflammatory response which is vital for embryo support. They showed that in decidualising ESCs from RMC women, a chaotic auto-inflammatory response, occurs as result of deregulated activation of IL-33/ST2L/sST2 pathways. This protracted anti-inflammatory response causes a prolonged implantation window and thereby disables natural embryo selection.

1.4.3.3 Migratory response of decidualised ESCs to embryo quality

Once the blastocyst breaches the luminal epithelium and enters the stromal compartment, the trophoblastic shell of the conceptus forms the floating and anchoring villi. The extravillous trophoblast (EVT) cells originate from the cell columns of these anchoring villi which then enter the decidua and inner third of the myometrium (reviewed in Brosens et al., 2002). The terminal spiral arteries are infiltrated by a sub-group of EVTs named as endovascular EVTs, which plug these vessels for several weeks before displacing the endothelial cells. This process also destroys the musculoelastic wall of the spiral arteries and thereby creating a low-resistance, high flow utero-placental circulation (Brosens and Gellersen, 2010). This invasive nature of EVTs which is intensely regulated by decidual cells is indispensable for embryo survival. *In-vitro* cell culture experiments offer intricate evidence on the intercellular cross-talk between the decidual and trophoblast cells. When blastocysts placed on a mono-layer of

decidualised ESCs in *in-vitro* co-culture experiments, the stromal cell motility increased at the contact zone with the expanding blastocyst and the cells move away from the blastocyst (Grewal *et al*., 2008; Grewal *et al*., 2010). The stromal cells acquire this enhanced migratory and invasive capacity only upon decidualisation which is further increased in response to trophoblast derived signals (Gellersen *et al*., 2010). The decidualisation is further augmented by the paracrine signals that come from trophoblast secretions and in response decidualised ESCs provide a positive environment for trophoblast expansion and invasion (Gonzalez et al., 2011). The trophoblast expansion into the decidua depends on the migratory capacity of the decidual cells and limiting these regulatory pathways that control motility, cyto-skeletal reorganisation and focal adhesion severely affect blastocyst expansion (Grewal et al., 2008; Grewal et al., 2010). In transwell migration assays, Gellersen et al. (2013) demonstrated the reciprocal attraction between decidual cells and the trophoblast, and suggested that this migration occurs as a result of active signalling between the two. They also revealed that the trophoblast spheroid expansion was greater on a monolayer of decidualised ESCs, which is further enhanced by a combination of implantation factors when compared to co-culture of trophoblast with undifferentiated ESCs. Decidual cell motility is controlled by various local factors such as heparin binding EGF-like growth factor (HB-EGF) and platelet derived growth factor (PDGF)-BB, and trophoblast-derived factors such as PDGF-AA and many other chemotactic factors yet to be identified (Schwenke et al., 2013).

Further *in-vitro* studies have shown that the decidualised ESCs from fertile women adjust their migratory activity in response to the quality of an embryo. Weimer et al., (2012) used well designed migration assays to demonstrate the migratory response of decidualised ESCs from RM women in the presence of trophoblast spheroids of different sizes and day 5 embryos of different developmental potential. The ESCs from women with RM more actively migrated towards the trophoblast spheroid when compared to ESCs from control women. The migration of ESCs from RM women in the presence of a developmentally incompetent embryo was significantly more when compared to the migration in the absence of an embryo. This migration was comparable to the migration of the ESCs from RM women to good quality embryo and also from fertile control to good quality embryos (Figure 1.3). These findings emphasise the increased migratory responsiveness of the decidualised ESCs from RM women towards EVT signals and their inability to differentiate developmentally competent embryos from the compromised ones.

To summarise, an effective endometrial transformation is compulsory for a successful implantation, which ensures that the stromal compartment acquires a receptive phenotype and also enables embryo recognition and selection. Failure of the ESCs to express this vital characteristic may lead to rapid conceptions, but allows repeated pregnancy failures in keeping with the 'selection failure' hypothesis. The decidual ESCs actively choose a good quality embryo for implantation and restrict their migratory activity for a developmentally incompetent embryo. The inability of decidual ESCs of RM

women to identify and to adjust migratory response to good and poor quality embryos may reinforce the concept of an impaired natural selection process.

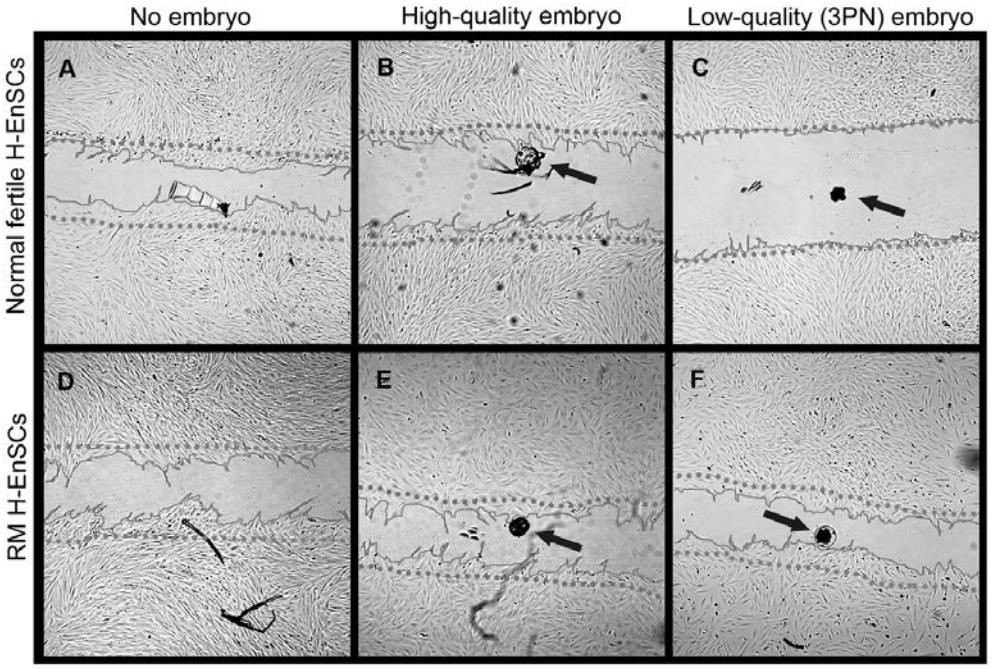


Figure 1.3: The migration zone after adding a high-quality, low-quality or no embryo. The migratory response of decidualized H-EnSCs from normally fertile (A–C) and RM women (D–F) was analyzed in absence of a human embryo (A and D), in presence of a high-quality embryo (B and E) or a low-quality embryo (C and F). Phase contrast pictures were taken 18 hours after creating the migration zone. The dotted line represents the front of the migration zone directly after its creation. As a reference for the position of the embryo, the bottom of the plate was marked. The arrows indicate the position of the embryo. All pictures were taken with 25x magnification. (From Weimer et al., (2012) Endometrial Stromal Cells of Women with Recurrent Miscarriage Fail to Discriminate between High- and Low-Quality Human Embryos. PLoS One 7(7): e41424.

1.4.4 Endometrial Leucocytes:

Though the fetus inherits both maternal and paternal histocompatibility antigens it still does not get rejected by the maternal tissues and this hemi-allogenic fetus survives within the maternal uterus as a result of an exceptionally structured and a bi-directional immunological association shared by the two. Various key factors in the peri-implantation endometrium are likely to play a major role in the immune success of normal pregnancy and the major responsibility lies with the endometrial leucocytes, which establish maternal immunologic adaptations to fetal antigens and by maintaining tissue integrity.

In the proliferative phase, the stratum functionalis of the endometrium has few scattered uterine leucocytes, mainly natural killer (uNK) cells, T cells and macrophages. These cells account for around 10% of the stromal cells and contribute to the majority of immune cell types in the endometrium (Bulmer *et al*., 1991a). Subsets of leucocytes and their phenotypes are mainly identified by flow cytometry analysis of surface antigens or by immunohistochemistry. The CD45 antigen is common to all endometrial leucocytes.

1.4.4.1 T-cells

The T cell density in the proliferative stage is about 45% of the leucocytes and numbers remains the same throughout the secretory phase and in pregnancy (Vassiliadou and Bulmer, 1996), but as substantial numbers of uNK cells

migrate into the endometrium in the peri-implantation period, the number of T cells relatively decrease in number. CD3 is a marker for all T cells that comprise both CD4 (T helper) and CD8 (cytotoxic T cell).

$CD8^+$ T lymphocytes are significantly reduced among RM women compared to normal controls. Those women with RM who have normal concentration of $CD8^+$ cells are subsequently more likely to have successful pregnancies while those with abnormal expression have an increased risk of subsequent miscarriages (Lachapelle *et al.*, 1996). Quenby *et al*., (1999) reported that endometrial biopsies from women with RM show a significantly increased expression of $CD4^+$ positive cells in the pre-implantation endometrium compared to fertile controls whereas there is no difference in the concentration of $CD3^+$ and $CD8^+$ cells. Those women who had a subsequent miscarriage had significantly more $CD4^+$, and $CD8^+$ cells. Several studies have reported no difference in decidual $CD3^+$ cells in RMC women when compared to normal early pregnancy (Quack *et al*., 2001). Reduced numbers of $CD4^+$ $CD25^{bright}$ regulatory T lymphocytes in the decidua is found in women with unexplained RM when compared with controls (Yang *et al*., 2008).

1.4.4.2 The endometrial macrophages

The macrophages are mononuclear phagocytic cells that are involved in both innate and humoral immunity. They have a varied distribution and are present in both stromal and epithelial compartments. The macrophage density significantly

increases in the late secretory phase of the menstrual cycle and in early pregnancy at the site of implantation (Bulmer *et al*., 1991b). They are the second most abundant leucocytes in the endometrium and decidua contributing to 20% of all decidual leucocytes (Bulmer *et al*., 1988). The endometrial macrophages are identified by their expression of CD14 and CD68 antigens.

The exact role of macrophages in the peri-implantation endometrium is not fully understood. It is thought that they may play a role in apoptosis of unwanted cells and clearing up of cell debris to maintain cellular homeostasis during implantation (reviewed in Mor and Abrahams, 2003). *In-vitro* studies suggest that macrophages are in close proximity to spiral arteries and they may have a role in the regulation of trophoblast invasion and transformation of spiral arteries, but their exact *in-vivo* function remains unknown (reviewed in Bulmer *et al*., 2010). A recent study by Li *et al*. (2009) demonstrated that the HLA-G homodimer induced the production of potential pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α from macrophages and uNK cells. A larger secretion of these cytokines was from macrophages and this observation reinforces the role of macrophages in spiral artery remodelling. Macrophages can also produce macrophage colony stimulating factor, granulocyte colony stimulating factor and TNF-α.

There are not many studies that have looked at the association between decidual macrophages and RM, the focus being mainly on uNK cells. Quenby *et al*. (1999) reported an increase number of macrophages in luteal phase

endometrium whereas Quack *et al*. (2001) did not find any differences in the macrophages between RM women and controls.

1.4.4.3 The uterine natural killer cells

uNK cells are the most abundant leucocytes in the decidual stroma and account for 70-80% of the decidual leucocyte population. The uNK cells are large lymphocytes (~45 µm diameter) with prominent cytoplasmic granules which contain lytic proteins and with a kidney shaped nucleus. They are also known as uterine large granular lymphocytes. The uNK cells are identified by their expression of CD56 antigen at high concentration ($CD56^{bright}$) and lack CD16, which is found on the most peripheral NK cells. Thus they are $CD56^{bright}$ $CD16^{-}$ in distinction to the most of the peripheral blood NK cells which are $CD56^{dim}CD16^{+}$ (Starkey et al., 1988). A subgroup of peripheral NK cells has a similar phenotype as uNK cells ($CD56^{bright}$ $CD16^{-}$), but a large majority are $CD56^{bright}$ $CD16^{+}$ (Lanier et al., 1983). The uNK cell density significantly increases following ovulation and continues to increase until a few days before menstruation (King et al., 1989). The uNK cells peak in density in the first trimester of pregnancy (Starkey et al., 1998).

The changes in the immune environment in the endometrium are thought to be equally important as compared to stromal decidualisation for a successful implantation and maintenance of pregnancy. In humans, the decidual process creates maternal immunologic tolerance to fetal antigens, thereby ensuring

protection of conceptus. Differentiating endometrial stromal cells have a function to regulate different immune cell density in the peri-implantation period and cause an influx of macrophages and uNK cells. The decidual stromal cells secrete interleukins (IL-11 and IL-15), which cause proliferation and differentiation of uNK cells in the decidualising stromal compartment (Dimitriadis *et al*., 2005).

The actual function of these uNK cells around implantation and early pregnancy remains debated and numerous questions on their origin and function continue to be unanswered. Previously, it was thought that they might facilitate implantation of the blastocysts, including those with aneuploidy and thus contributing to early pregnancy failures. There is now a good body of evidence to suggest that uNK cells are involved in promoting decidualisation and are involved in regulating fetal trophoblast invasion by their production of crucial cytokines, chemokines and growth factors such as IL-8, IP-10, TNF-α, TGF-β1 and IFN-γ (reviewed in Bulmer *et al*., 2010). They also induce angiogenesis and vascular remodelling in the decidua by their ability to secrete an array of angiogenic growth factors (Hanna *et al*., 2006). Appropriate development and maintenance of decidua and maternal arteries is impaired in mice when uNK cells are absent from the peri-implantation endometrium. The uNK cell deficient mice have decidual cell degeneration, endothelial cell alteration (Greenwood *et al*., 2000) and poor spiral arteries (van den Heuvel *et al*., 2005).

Kuroda *et al*. (2013) demonstrated that decidualisation was associated with a significant induction of 11-β hydroxysteroid dehydrogenase type 1 enzyme

(11βHSD1), that catalyses the conversion of inactive cortisone to active cortisol, in a time-dependent manner. Inhibition of 11βHSD1 decreases cortisol synthesis and thus selectively affects expression of decidual marker genes. This defective decidualisation was found to be associated with women with a high uNK cell density of more than five percent in the peri-implantation endometrium as assessed by immunohistochemical methods.

Recruitment and proliferation of leucocytes in the endometrium is an important transformation that occurs as a part of decidualisation and altered uNK cell function and density has been proposed to play a major role in reproductive failures. Many studies have found an increased uNK cell density in the endometrium of women with RM (Lachapelle *et al*., 1996; Quenby *et al*., 1999, 2005, 2009; Tuckerman *et al*., 2007). A systematic review suggested no conclusive evidence of high levels of uNK cells in predicting subsequent miscarriage in women with idiopathic RM (Tang *et al.*, 2011). Quenby *et al.* (2005), in their prospective study investigated the effects of steroids on uNK cells and successfully demonstrated a marked decrease in the uNK cell density in women with RM after three weeks of treatment with prednisolone. A pilot double blind randomised controlled trial designed to assess the feasibility of screening women with idiopathic RM for high uNK density and randomising to prednisolone or placebo found an increase in live birth rate and decrease in miscarriage in the prednisolone group when compared to placebo group, (LBR: RR 1.5, 95% CI 0.8-2.9 and for miscarriage RR 0.67, 95% CI 0.4 – 1.3) (Tang *et al*., 2013). In this trial, women with very high uNK density of more than ten

percent appeared to have the worst outcome when compared to those with uNK cell density of five to ten percent.

In summary, the immune system plays a dominant role in the normal biology of human endometrium. The recruitment and proliferation of uNK cells depends on effective decidualisation and high uNK density is associated with perturbed decidualisation. Though decidualisation causes influx of uNK cells into the endometrium, their appropriate regulation is vital as high density uNK cells in the peri-implantation endometrium appears to contribute to pregnancy complications including RM. Steroid treatment has been shown to be beneficial in reducing the uNK cell density. The role of other leucocytes in decidualisation and implantation remain unclear.

1.4.5 Endometrial mesenchymal stem cells

Spontaneous endometrial stromal decidualization is an outstanding feature in humans, shared with only a few other mammalian species, and is also responsible for the menstrual shedding of the endometrium in the absence of a pregnancy. The human endometrium has remarkable regenerative capacity (Gargett et al., 2012) and with an ability to grow to 5-7 mm thickness from an initial 0.5-1 mm just after menstruation (McLennon and Rydell, 1965). The endometrium cyclically proliferates, differentiates, sheds and then regenerates in response to ovarian steroids for about 400 times in a woman's reproductive lifespan (Jabbour et al., 2006; Brosens and Gellersen, 2006). Endometrial

regeneration also occurs following resection of the endometrium after pregnancy and in women taking hormone replacement therapy after menopause (Gargett. 2006; Gargett. 2007). Adult stem cells, derived from postembryonic cell lineages and with their ability of cellular production, are present in highly regenerative bone marrow, the epidermis and intestinal epithelium are responsible for a new tissue growth in these organs (reviewed in Gargett and Masuda, 2010). The adult stem cells have an important role in maintaining tissue homeostasis by providing replacement cells lost through cellular turnover and following tissue damage (Gargett and Healy, 2011). These adult stem cells that are identified in the stratum basalis of the endometrium do not differ between phases of the menstrual cycle and are also present in the atrophic endometrium of post-menopausal women and in inactive endometrium of women taking hormonal contraceptive pills (Schwab et al., 2005). Gargett and Masuda, (2010) reviewed the available evidence on eMSCs and found that the endometrial stroma harbour undifferentiated multipotent eMSCs which are phenotypically and characteristically similar to the ones found in bone marrow and adipose tissue, but they are absent in other reproductive tissues such as myometrium, Fallopian tubes and uterosacral ligaments.

The origin of these stem cells in the endometrium remains unanswered. It is possible that the eMSCs are derived from residual fetal stem cells (Gargett, 2007), but there is also some evidence to suggest that bone marrow-derived cells may also add to the pool of resident adult stem cells in the endometrium (Ikoma et al., 2009). In mouse studies, Du and Taylor (2007) have demonstrated that the stem cells derived from the bone marrow are present in

the endometrium. Using a transgenic mouse, Bratincsak et al., (2007) showed that CD45- positive haematopoietic stem cells are present in the endometrial epithelium, which are responsible for contributing to more than eighty percent of the endometrial epithelial cells in pregnancy. eMSCs are a subpopulation of pericytes found in the peri-vascular regions of the endometrial stroma, both in the basalis and functionalis layers (Schwab and Gargett, 2007).

Clonogenecity, the ability of single cells to form colonies when plated at very low densities, has traditionally been used to characterize stem cells populations derived from multiple adult tissue types (Chan et al., 2004).

Cancer stem cells, which are identical to adult stem cells in their key properties, but not in function as they are not regulated by the stem cell niche (Pardal et al., 2003) – anatomic structure where adult stem cells reside (Li and Xie, 2005), are implicated in breast cancer, acute myeloid leukaemia, prostate cancer, glioblastoma and endometrial cancer (Pardal et al., 2003; Kato et al., 2008; Hubbard et al., 2009). The eMSCs are also associated with the development of endometriosis (reviewed in Sasson and Taylor, 2008) and adenomyosis (Chen et al., 2010). MSCs, particularly the bone marrow derived ones, are a well-established source of cells in regenerative medicine and are increasingly being used in clinical trials for the treatment of heart disease, stroke, cartilage repair and spinal cord injury (reviewed in Gargett and Masuda, 2010).

1.5 Project hypotheses and objectives:

In this thesis, to explore the effects of obesity on the endometrium, I hypothesise the following:

1. Obese women have an increased risk of first trimester miscarriage and the pregnancy loss pattern is different in obese women when compared to normal weight women as a result of the negative effects of obesity.

2. Obese women with recurrent miscarriage have a significantly shorter time to pregnancy when compared to normal weight women with recurrent miscarriage secondary to perturbations in the peri-implantation endometrial development.

3. The leucocytes density in the peri-implantation endometrium, the immune cells which are likely to play a role in reproductive success, is significantly different in obese women when compared to that of normal weight women.

4. The abundance and clonogenic efficiency of endometrial mesenchymal stem-like cells, which play an important role in endometrial regeneration, are impaired in the peri-implantation endometrium of obese women.

5. The decidual programming of the peri-implantation endometrium, an essential transformation for a successful implantation, is perturbed in an obesogenic environment.

In order to study my hypotheses, I set out the following specific objectives:

1. To evaluate the early pregnancy reproductive outcome of women who attended a tertiary referral Implantation Clinic with various reproductive failures, particularly looking at early pregnancy loss patterns in obese women comparing them to normal and overweight women.

2. To analyse pregnancy loss pattern and time to pregnancy intervals in obese women with recurrent miscarriage and compare them to normal weight women with recurrent miscarriage.

3. To define the peri-implantation endometrium in obese women and compare it to normal weight women, particularly looking at:
    a. The sub-epithelial uterine natural killer cell and macrophage density by immunohistochemical studies.
    b. Endometrial mesenchymal stem cell density and function using magnetic beads separation and in-vitro colony-forming assay
    c. In-vitro endometrial stromal cell differentiation and analysis of decidual markers expression in artificial obesogenic environment

CHAPTER 2:

SUBJECTS, MATERIALS AND METHODS

2.1 Ethical approval

This research was conducted at and supported by Biomedical Research Unit in Reproductive Health, a joint initiative between University Hospitals of Coventry and Warwickshire NHS Trust and the University of Warwick. Ethical approval for this study was obtained from the National Research Ethics - Hammersmith and Queen Charlotte's Hospital Research Ethics Committee (1997/5065) and the research sponsorship for this study has been transferred from Imperial College, London to the University of Warwick. The study has also been approved by the University Hospitals Coventry and Warwickshire Research and Development department, and written informed consent was obtained from all participants, in accordance with the guidelines in The Declaration of Helsinki 2000.

2.2 Patient selection:

The subjects were recruited for the study from the tertiary referral Implantation clinic, a dedicated endometrial research clinic at the Centre for Reproductive Medicine, University Hospitals of Coventry and Warwickshire NHS Trust for women with recurrent miscarriage or recurrent IVF treatment failure. The women were either referred from IVF and recurrent miscarriage clinics around the United Kingdom or the women contacted the clinic directly. At the initial consultation, the medical history that included details of previous pregnancy outcome for each pregnancy and details of time to pregnancy intervals was recorded by a medical staff from these women. The early pregnancy losses

(EPL) in the first trimester were classified as (i) Biochemical pregnancies losses (BPL): where the pregnancy was diagnosed by either serum or urine b-hCG without an ultrasound evaluation and the serial results suggesting a pregnancy loss, (ii) Empty gestational sac losses (EGSL): where an intra-uterine pregnancy sac was visualised on ultrasound with or without yolk sac, but without an embryo (iii) Fetal losses (FL): where an intrauterine pregnancy was demonstrated on ultrasound, with an embryo, but without cardiac activity or at least two ultrasound scan assessments with at least 7 days difference suggesting intrauterine pregnancy with an embryo, but with no cardiac activity, (iv) Recurrent miscarriage (RM): three or more consecutive pregnancy loss less than 24 weeks of gestation.

All subjects who attended the clinic underwent anthropometric measurements and the BMI or Quetelet index was calculated using the standard definition of “weight in kilogrammes divided by the square of height in meters”. The WHO classification was used to categorize women into three groups: Normal (BMI: 19.0 – 24.9 kg/m2), Overweight (BMI: 25 – 29.9 kg/m2) and Obese (BMI ≥ 30 kg/m2).

The women who underwent endometrial biopsy were advised to use ovulation prediction kits to detect a surge in LH and were instructed to contact the clinic co-ordinator for an appointment 5 to 10 days after the pre-ovulatory LH surge.

2.3 Endometrial sampling:

The endometrial biopsies were obtained from patients in the peri-implantation period, 5 to 10 days after the pre-ovulatory LH surge. The procedure was explained to the women and a written informed consent was obtained in accordance with the guidelines in The Declaration of Helsinki, 2000. They were also warned of possible cramp-like lower abdominal pain during and after the procedure and that some may experience vaginal spotting or bleeding.

A trans-vaginal ultrasound scan was carried out to identify the uterine shape, size, version, endometrial thickness and to rule out any uterine and endometrial pathology. The women were placed on an examination table with feet and legs supported as for a pelvic examination. The cervix was visualised using a bi-valve, self-retaining Cusco's speculum. A disposable endometrial sampler (Wallach Endocell^{TM} sampler, Wallach Surgical Devices Inc., Trumbull, USA) was gently introduced into the uterine cavity until the fundus was reached. A tenaculum or vulsellum was used to the upper lip of the cervix to steady the cervix and uterus if a difficulty was encountered at initial insertion of the endometrial sampler. The inner piston of the sampler was drawn back creating suction and the endometrial sample was obtained starting from the fundus and moving downwards to the internal cervical ostium, rotating it while being removed from the cavity. One half of the sample was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at pH 7.4 for 24 to 48 hours, then processed in a tissue sampler overnight and embedded in paraffin

for immunohistochemistry. The other half of the sample was placed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Life Technologies Ltd., Paisley, UK) and transported immediately to the laboratory for isolation of primary human endometrial stromal cell culture.

For some women it was difficult to obtain the endometrial biopsy sample in the clinic and they underwent a diagnostic hysteroscopy and endometrial biopsy under a general anaesthetic.

2.4 Immunohistochemistry:

2.4.1 Materials:

1. Adhesive microscope slides - Leica Xtra® adhesive slides, Leica Microsystems (UK) Ltd, Milton Keynes, UK

2. Distilled water

3. Hematoxylin (<0.1%) - Leica Microsystems (UK) Ltd, Milton Keynes, UK

4. Isopropyl alcohol (IPA) – 100%, 90% and 70%

5. Novocastra™ Protein Block - 0.4% Casein in phosphate-buffered saline, with stabilizers, surfactant, and 0.2% Bronidox L as a preservative, Leica Microsystems (UK) Ltd, Milton Keynes, UK

6. Novolink™ Polymer (Anti-rabbit Poly-HRP-IgG (<25µg/mL) containing 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950) - Leica Microsystems (UK) Ltd, Milton Keynes, UK

7. PAP hydrophobic pen - abcam®, Cambridge, UK

8. PickCell 2100 steam antigen retrieval unit - PickCell Laboratories, Leiden, the Netherlands

9. Phosphate buffered solution (PBS)

10. Post-primary (Rabbit anti mouse IgG (<10 µg/mL) in 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950) - Leica Microsystems (UK) Ltd, Milton Keynes, UK

11. Primary antibody (Monoclonal Mouse anti-Human CD56 clone 123C3) - Dako UK Ltd, Ely, Cambridgeshire, UK

12. Primary antibody (Novocastra™ Lypholized Mouse Monoclonal antibody CD14) - Leica Microsystems (UK) Ltd, Milton Keynes, UK

13. Rotary Microtome - Leica RM 2235, Leica Microsystems (UK) Ltd, Milton Keynes, UK

14. Sodium citrate antigen retrieval solution - Leica Microsystems (UK) Ltd, Milton Keynes, UK

15. Tris-buffered saline plus 0.05% tween20 (TBST)

16. Xylene

17. 3% (v/v) hydrogen peroxide - Leica Peroxidase Block, Leica Microsystems (UK) Ltd, Milton Keynes, UK

18. 3, 3' – diaminobenzedine (DAB) (prepared from DAB Chromogen: 1.74% w/v 3,3' - diaminobenzidine, in a stabilizer solution and Novolink™ DAB Substrate Buffer (Polymer): Buffered solution containing ≤0.1% hydrogen peroxide and preservative) - Leica Microsystems (UK) Ltd, Milton Keynes, UK

2.4.2 Slide preparation

The endometrial sample was fixed in 4% paraformaldehyde in PBS at pH 7.4 for 24 to 48 hours, then processed in a tissue sampler overnight and embedded in paraffin. 3 microns thick sections of the paraffin embedded biopsies were cut on a rotary microtome. The sections were floated in a warm water bath (45^0C) before being picked up onto adhesive microscope slides and allowed to drain. The slides were placed into an oven at 65^0C for a minimum of an hour, preferably overnight, to ensure sections adhere to slides. De-waxing of the paraffin sections was done by placing them into staining vessels containing Xylene for 5 minutes, twice, then twice in 100% IPA for 5 minutes each, in 90% IPA for 5 minutes, in 70% IPA for 5 minutes and finally in Distilled Water for 5 minutes. The sections were placed in optimal pH buffer (Sodium citrate antigen retrieval solution, buffer pH 6) for antigen retrieval in PickCell 2100 steam antigen retrieval unit for 2 hours. Margins were drawn on either end of the slide, bracketing tissues, using a PAP hydrophobic pen to limit the amount of reagents used.

The sections were washed twice with TBST for 5 minutes each time. Endogenous peroxidase activity was blocked using 3% (v/v) hydrogen peroxide for 10 minutes. The sections were washed twice with TBST for 5 minutes each, followed by incubate in Novocastra™ Protein Block for twenty minutes to an hour to reduce non-specific binding of primary and polymer.

2.4.3 Staining for CD56/CD14 antigens:

Primary antibody was applied to the above prepared slides in a dilution of 1/250 and kept overnight at 4^0 C or at room temperature for 1 hour. The slides washed twice with TBST for 10 minutes and secondary antibody was applied for 30 minutes at room temperature to detect mouse antibodies. The slides were washed twice with TBST for 5 minutes and a polymer (Novolink™ Polymer) was applied for 30 minutes at room temperature which recognizes rabbit immunoglobulins, post-primary and any tissue bound rabbit primary antibodies. The sections were then washed twice in TBST for 5 minutes and incubated with DAB for 7 minutes. Reaction with the peroxidase produces a visible brown precipitate at the antigen site. The slides were then washed in distilled water for 5 minutes and haematoxylin was applied for 30 seconds to a minute for counterstaining. The slides were then rinsed in distilled water and washed in TBST for 5 minutes to blue up haematoxylin. The cells were dehydrated and mounted.

2.4.4 Analysis and interpretation of the slides:

The images were processed and viewed using 'Panoramic Viewer' (3D Histech Ltd, Budapest, Hungary). The pictures were captured at 40 x magnification in 5 randomly selected high power fields and the subepithelial region, avoiding glandular areas, were analysed for uterine leucocyte (uNK cell or macrophage) density. The blue stained stromal cells and brown stained uNK cells (CD56+) and macrophages (CD14+) were counted using the software 'ImageJ' and 'point picker plug-in tool' (National Institutes of Health, Bethesda, MS, USA). An average of 500 cells per high power field and hence approximately 2500 cells were counted from an endometrial biopsy. The cell density was calculated using the formula total number of uterine leucocytes divided by total number of stromal cells and then multiplied by 100.

2.5 Endometrial mesenchymal stem-like cell isolation and cloning efficiency

2.5.1 Materials:

Antibiotic-antimycotic solution (1%) - Invitrogen, Paisley, UK

Anti-PE MicroBeads - Miltenyi Biotec. Bisley, UK

Basic fibroblast growth factor - Merck Millipore, Watford, UK

Cell strainer (100 µm and 40 µm) - Fisher Scientific, Loughborough, UK

Collagenase (0.5mg/ml) - Sigma-Aldrich, Gillingham, UK

Deoxyribonuclease type I (0.1mg/ml) - Roche, Burgess Hill, UK

Dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) (10%)

Distilled water

DMEM/F-12 medium - Invitrogen, Paisley, UK

Estradiol - Sigma-Aldrich, Gillingham, UK

Ficoll-Paque PLUS - GE Healthcare, Little Chalfont, UK

Fibronectin (10ug/ml in PBS)

Haematoxylin

Insulin - Sigma-Aldrich, Gillingham, UK

L-glutamine - Invitrogen, Paisley, UK

MACS separator - Miltenyi Biotec. Bisley, UK

Magnetic Bead Buffer: containing phosphate buffered saline (PBS), pH 7.2, and 0.5% bovine serum albumin (BSA) by diluting MACS® BSA Stock Solution 1:20 with autoMACS® Rinsing Solution - Miltenyi Biotec, Bisley, UK.

MS columns - Miltenyi Biotec. Bisley, UK

Phycoerythrin (PE) conjugated Anti-SUSD2 (W5C5) antibody - BioLegend, London, UK

2.5.2 Isolation of single cell suspensions of endometrial stromal cells:

The endometrial tissue was collected on a petri-dish, washed in DMEM/F-12 medium and the fluid around the tissue was removed completely. The endometrial tissue was finely minced using a sterile blade for 5 minutes and enzymatically digested with collagenase (0.5mg/ml) and deoxyribonuclease type I (0.1mg/ml) in 10ml of 0% medium (DMEM/F-12 without DCC, L-glutamine and antibiotics) for 1 hour at 37^{o}C. The dissociated cells were filtered through a sterile 100µm cell strainer on 50 ml tube to remove large undigested tissue and then through a 40µm cell strainer to remove glandular clumps. Most of the stromal cells and blood cells, present as a single cell suspension, passed through the cell strainer, whereas the undigested fragments, mostly comprising of glandular clumps, were retained on the strainer. 10ml of 10% culture medium (DMEM/F-12 with 10% DCC-FBS, 1% L-glutamine, 2 µg/ml insulin, 1nM estradiol and 1% antibiotic-antimycotic solution) was added onto the cell strainer and centrifuged at 1200rpm for 5 minutes. The stromal cell pellet was resuspended in 8 ml of 10% culture medium.

Stromal single cell suspensions were then layered over Ficoll-Paque PLUS to produce two layers and centrifuged at 1500rpm for 15 minutes to remove erythrocytes. The cells at the medium/Ficoll-Paque PLUS interface, mainly containing stromal cells, were carefully aspirated by pipetting, washed twice

with 10% culture medium, centrifuged at 1200rpm for 5 minutes to remove some Ficoll-paque, and then subjected to magnetic bead separation.

2.5.3 Magnetic cell separation:

Magnetic bead separation was performed according to the manufacturer's instruction (Miltenyi Biotec, Bisley, UK). Freshly isolated endometrial stromal cell number was determined and the cell suspension was centrifuged at 1200rpm for 5 minutes followed by aspiration of the supernatant. The cell pellet was resuspended in Magnetic Bead Buffer (up to 1×10^6 cells/100µl) and then was incubated with PE conjugated Anti-SUSD2 (W5C5) antibody (5µl/1×10^6 cells) on ice in the dark for 20 minutes. The cells were washed by adding 1ml of Magnetic Bead Buffer per 10^7 cells and centrifuged at 1200 rpm for 5 minutes to remove unbound primary antibodies. Then cell suspensions (up to 1×10^7 cells/80µl of Magnetic Bead Buffer) were incubated with Anti-PE MicroBeads (20µl/1×10^7 cells) on ice in the dark for 20 minutes. The cells were washed by adding 1ml of Magnetic Bead Buffer per 10^7 cells and centrifuged at 1200 rpm for 5 minutes at 4^0 C. The cell suspensions (up to 1×10^8 cells/500ul of Magnetic Bead Buffer) were applied onto the MS columns in a magnetic field (MACS separator), followed by washing with 500ul of Magnetic Bead Buffer three times. While most unlabelled ($W5C5^-$) cells passed through the column, magnetically labelled $W5C5^+$ cells were mostly retained on the column. The columns were removed from the magnetic field and $W5C5^+$ cells were flushed out by firmly pushing the plunger with 1ml of Magnetic Bead Buffer. The magnetic cell

separation steps were repeated using a new MS column to increase the purity of the magnetically labelled cells.

2.5.4 In-vitro colony forming unit (CFU) assay:

60mm culture dishes were coated with 2 ml of fibronectin for at least 20 minutes. The excess fibronectin was removed and replaced with growth medium containing DMEM/F12 containing 10% DCC-FBS, 1% L-glutamine, 1% antibiotic-antimycotic solution, insulin (2µg/ml), estradiol (1nM) and basic fibroblast growth factor (10ng/ml). Freshly isolated $W5C5^+$ and $W5C5^-$ cells were seeded onto culture dishes at a clonal density of 50 cells/cm^2 per each sample. The first medium change was after the first 7 days. Subsequently, media was changed twice in the next week. Colonies were monitored microscopically to ensure that they were derived from single cells. Cultures were terminated at 15 days, washed thrice in PBS and were fixed in 10% formalin (4% formaldehyde) for 10 minutes. After washing the cultures for three times with PBS, they were incubated with hemotoxylin for 4 minutes for staining. The plates were washed in distilled water, dried, and photographed to count the colonies using software 'ImageJ' (National Institutes of Health, Bethesda, MS, USA). Clusters of ≥ 50 cells were counted and the cloning efficiency (CE) was determined from the formula: cloning efficiency (%) = (number of colonies/number of cells seeded) $\times$ 100.

2.6 Endometrial stromal cell differentiation

2.6.1 Materials:

Antibiotic-antimycotic solution (1%) - Invitrogen, Paisley, UK

Collagenase (0.5mg/ml) - Sigma-Aldrich, Gillingham, UK

Deoxyribonuclease type I (0.1mg/ml) - Roche, Burgess Hill, UK

Dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) (10%)

Distilled water

DMEM/F-12 medium - Invitrogen, Paisley, UK

Estradiol - Sigma-Aldrich, Gillingham, UK

Insulin - Sigma-Aldrich, Gillingham, UK

L-glutamine - Invitrogen, Paisley, UK

gDNA Wipeout Buffer, 7x – QIAGEN, Manchester, UK

RNA Stat-60 – AMS Biotechnology, Abingdon, UK

NanoDrop spectrometer - Thermo Scientific, Wilmington, USA.

Reverse transcriptase enzyme - QIAGEN, Manchester, UK

RT buffer 5x - QIAGEN, Manchester, UK

RT primer mix - QIAGEN, Manchester, UK

SYBR® Green PCR master mix – Applied Biosystems, Paisley, UK

7500 Real-time PCR system - Applied Biosystems, Paisley, UK

Optical 96-well reaction plate - Applied Biosystems, Paisley, UK

Optical adhesive film – Alpha laboratories, Hampshire, UK

2.6.2 Primary cell culture of human endometrial stromal cells:

The endometrial tissue was collected on a petri-dish, washed in DMEM/F-12 medium and the fluid around the tissue is removed completely. The endometrial tissue was finely minced using a sterile blade for 5 minutes and enzymatically digested with 100µl collagenase (0.5mg/ml) and 100µl deoxyribonuclease type I (0.1mg/ml) in 10 ml of the medium (DMEM/F-12 without DCC, L-glutamine and antibiotics) for 1 hour at 37^{o}C. 10 ml of the maintenance medium, a mixture of DMEM/F12 containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), antibiotic and antimycotic solution, and 1% L-glutamine was added to deactivate collagenase and the cells were separated by centrifugation at 1200 rpm for 5 minutes. The cell pellet was resuspended in 10% maintenance medium and the separated endometrial stromal cells (ESCs) were passaged in T75 flasks (13 ml of 10% maintenance medium). The proliferating ESCs were cultured in maintenance medium, which was changed every two days, until confluence.

2.6.3 Decidualisation of endometrial stromal cells in vitro:

The confluent cells were passaged by trypsinisation into 6-well plates and further cultured in maintenance medium until confluence. When the cells reached 70-80% confluence, 2% maintenance medium (DMEM/F-12 with 2% DCC-FBS) was used to synchronise cells. Confluent monolayer were treated for decidualisation in phenol red-free DMEM/F12 containing 2% DCC-FBS, with 8-bromo-cAMP (0.5 mM) and MPA (10^{-6} M) for 0, 4 and 8 days. This medium was changed every 48 hours. Supernatants were collected and frozen cells harvested for mRNA analysis.

2.6.4 Total RNA extraction:

200µL of RNA Stat-60 was added to each well. Cell lysates were scraped using scrapers and were transferred to pre-chilled RNase-free eppendorfs. 80µL of ice-cold chloroform was added to each eppendorf ($1/5^{th}$ of the Stat-60 used) and the contents were vigorously shaken. The samples were centrifuged in a cold room (4^0 C) at a maximum speed of 16000rpm for 30 minutes. The clear top layer was transferred into a second pre-chilled eppendorf and 200µL of ice-cold iso-propranolol was added (1/2 of the original Stat-60 volume). The contents were mixed by vortex and stored at -80^0 C for at least 30 minutes. The contents were centrifuged in a cold room (4^0 C) at maximum speed of 16000rpm for 30 minutes. The supernatant was carefully discarded and 1 ml of

70% ice-cold Ethanol (made up in nuclease free water) was added to the pellet. Following centrifugation in a cold room at a maximum speed of 16000rpm for 30 minutes, the supernatant was discarded and the pellet was allowed to air dry (2 minutes). 20µL of RNase-free DEPC water was added to dissolve the pellet and the RNA concentration was measured using NanoDrop spectrometer (Thermo Scientific, Wilmington, USA).

2.6.5 Reverse transcription and synthesis of complementary DNA (cDNA):

The template RNA mean concentration extracted from cell cultures (as described above) was calculated by NanoDrop spectrometer. Genomic DNA (gDNA) elimination reaction was done (Table) as gDNA contamination can lead to false positive results. The components were incubated for 2 minutes at 42°C, and then placed immediately on ice.

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA up to 1 µg*	Variable
RNase-free water	Variable
Total reaction volume	14 µl

Table 5.1: Components of gDNA elimination reaction.

The reverse transcription (RT) master-mix (MM) is prepared as in table 5.2. The components were mixed and then kept on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

Component	Volume/reaction
Reverse transcriptase enzyme	1 µl
RT buffer 5x	4 µl
RT primer mix	1 µl
Total reaction volume	6 µl

Table 5.2: Reverse transcription master-mix (RT MM) components.

Three templates were prepared for isolation of cDNA from the template RNA out of which two were controls (i) RT+ (ii) RT- and (iii) Control buffer (CB) or No RT control (Tables 5.3, 5.4 and 5.5).

Component	Volume/reaction
Template RNA - entire genomic DNA elimination reaction (Table 1)	14 µl
Reverse transcription master mix (Table 2)	6 µl
Total reaction volume	20 µl

Table 5.3: Reverse transcription reaction components for RT+ template

Component	Volume/reaction
gDNA elimination reaction without template RNA	14 µl
Reverse transcription master mix (Table 2)	6 µl
Total reaction volume	20 µl

Table 5.4: Reverse transcription reaction components for RT- template

Component	Volume/reaction
Template RNA - entire genomic DNA elimination reaction (Table 1)	14 µl
Reverse transcription master mix without RT enzyme, but replaced with water	6 µl
Total reaction volume	20 µl

Table 5.5: Reverse transcription reaction components for CB template (No RT control)

The above reactions were incubated for 30 minutes at 42°C to obtain cDNA, and then at 95°C for 3 minutes to inactivate RT enzyme. The reactions were placed on ice for real-time polymerase chain reaction (PCR).

2.6.6 Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR):

The PCR primer-master mix (PCR primer-MM) was prepared with the ratio of components as described in the table 5.6.

Component	Volume/reaction
Water	7.8 µl
SYBR® Green PCR master mix	10 µl
Primer Mix (Sum of each primer: forward + reverse)	1.2 µl
Total volume	19 µl

Table 5.6: PCR primer-MM components for real time RT-PCR

Specific primer pairs were designed using Primer3 software

(http://frodo.wi.mit.edu)

L19 sense: 5'-GCG GAA GGG TAC AGC CAA T-3'

L19-R antisense: 5'-GCA GCC GGC GCA AA-3'

Prolactin (*PRL*) sense: 5'-AAG CTG TAG AGA TTG AGG AGC AAA C-3'

PRL antisense: 5'-TCA GGA TGA ACC TGG CTG ACT A-3'

IGFBP1 sense: 5'-CGA AGG CTC TCC ATG TCA CCA-3'

IGFBP1 antisense, 5'-TGT CTC CTG TGC CTT GGC TAA AC-3'

In an optical 96-well reaction plate, 19 µl of the above mix was added to each well by reverse pipetting and 1 µl of the cDNA (RT+) or blank (RT- or CB or Water (No-template control – NTC)) was added into the relevant wells according to the template. An optical adhesive film was used to seal the optical 96-well reaction plate and centrifuged at 3000 rpm for 3 minutes. *mRNA* gene expression was measured on the PCR system. RNA input variances were normalized against the levels of the L19 housekeeping gene, which encodes a

ribosomal protein, using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001). All measurements were performed in duplicate.

2.7 Differentiation of endometrial stromal cells in the presence of supernatant from adipose tissue explants

The abdominal adipose tissue from the omentum was collected at caesarean section from normal weight women and obese women. The cleaned adipose tissue was dissected into small pieces and incubated in CMRL-1066 nutrient media (Sigma-Aldrich, Gillingham, UK) for 48 hours. The supernatant was collected and snap frozen (Obtained from Division of Metabolic and Vascular Health, Warwick Medical School, The University of Warwick)

Four sets of primary cell cultures of hESCs were established from endometrial biopsies and a confluent monolayer were treated with c-AMP and MPA for differentiation as described previously. The first group of cells (Control) were allowed to differentiate under normal conditions for 8 days. Three other groups of differentiating ESCs were treated with the culture media (CM group: CMRL 1066 nutrient media) (n=2), supernatant from adipose tissue explants of normal weight women (Normal) (n=3) and supernatant from the adipose tissue explants of obese woman (Obese) (n=3). *mRNA* gene expression for decidualisation markers (*PRL* and *IGFBP1*) were measured on day 8 as described previously and the relative expression of decidualisation markers from the CM, normal and obese groups were charted against the control group.

2.8 Statistical analysis:

Data was checked for normal distribution using histograms and the Kolmogorov-Smironov normality test with Lilliefors significance correction or Shapiro-Wilk normality test, as appropriate. Data are in mean ± SD, mean ± SEM and median ± IQR as required. Pearson's correlation or Spearman's correlation was used as appropriate for calculation of associations between variables. Data were analysed using ordinary one way ANOVA (post hoc analysis: Tukey HSD test) or Independent samples T test or Chi-Squared test or independent samples Kruskall-Wallis nonparametric test (± pair-wise comparison) or independent samples Mann-Whitney U nonparametric as appropriate to compare between the BMI groups.

Kaplan-Meier curves were constructed estimating the cumulative probability of a spontaneous pregnancy over time. The log-rank (Montel-Cox) test was used to test differences between the Kaplan-Meier curves for statistical significance.

All statistical analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, USA). $p < 0.05$ was considered significant.

CHAPTER 3:

HIGH BODY MASS INDEX AND FIRST TRIMESTER PREGNANCY LOSS PATTERN

3.1 Introduction

The commonest adverse outcome associated with pregnancy is miscarriage. An estimated 30% of the embryos are lost prior to implantation, a further 30% after implantation but before next menstruation and 10% miscarry clinically after a missed period (reviewed in Macklon et al., 2002) (Figure 3.1). Chromosomal aberrations appear to be common in the pre-implantation embryos which predispose to an increased risk of pregnancy losses. Out of the 23 good quality embryos examined by Vanneste et al (2009), only two were found to have normal karyotype in all blastomeres with nearly half of the embryos had no normal cells and the remaining embryos were mosaic for structural chromosomal imbalances. A further study that looked at more than 6000 embryos, many suitable for embryo transfer, suggested that only 44% of the embryos with best morphology and development were euploid in women under the age 35 and merely 21% were chromosomally normal in women aged 41 or more (Munné et al., 2007). However, the prevalence of abnormal karyotype of a pregnancy following a single sporadic miscarriage is 45% (95% CI: 38-52), which is not significantly different to that for a subsequent miscarriage following RM (39%, 95% CI: 29-50) (van den Berg et al., 2012).

Studies have reported that early stages of embryo development are susceptible to genetic disorders. However, the outstanding feature of early pregnancy is that of a ‘natural embryo selection’ – most of the aneuploid pregnancies (90%)

miscarry whereas 93% of karyotypically normal pregnancies continue (McFadden, 1989).

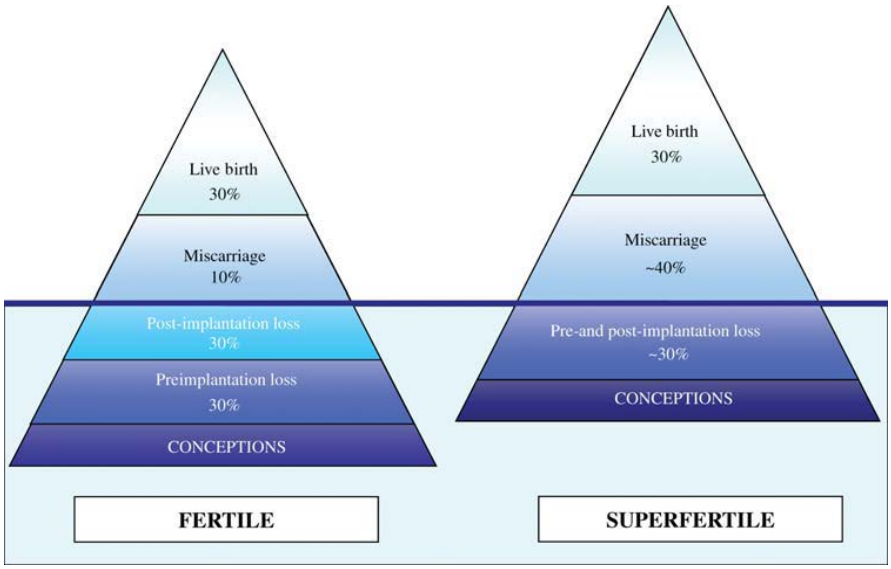


Figure 3.1: The embryo wastage iceberg in fertile and superfertile women. The embryo wastage icebergs give an overview of the outcome of conceptions in a normal fertile population and a specific subset of a superfertile population, representing 3% of all couples. The 'sea-level' in the figure distinguishes the pregnancies lost prior to the time of the missed menstrual period and clinically recognized pregnancy losses. (with permission from Oxford Journals: Teklenburg et al., (2010) The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod. 16(12):886-95.)

Epidemiological studies have indicated a robust association between increased maternal age and both spontaneous miscarriage and RM (Quenby and Farquharson, 1993; Nybo Anderson et al., 2000) indicating that embryo abnormality to be an important aetiological factor in miscarriage. While most of the embryo chromosomal abnormalities are as a result of oocyte aberrations associated with advanced maternal age (Robinson et al., 2001), there is also an association between abnormal sperm chromosome from the male partners of women experiencing RM (Egozcue et al., 2003) signifying the role of paternal factors in causing embryo abnormalities.

There is a lack of good quality evidence to suggest the effects of high BMI on early pregnancy outcome, but the evidence from observational studies, mostly retrospective, suggested that high BMI may be a risk factor for an increased risk of first trimester miscarriage and recurrent miscarriage (RM) in women who conceived spontaneously (Boots and Stephenson, 2011). Similarly, the impact of obesity on miscarriage rate after assisted reproduction technology (ART) remains less clear and inconsistent. However, a recent systematic review and meta-analysis of the existing literature, mainly retrospective studies, suggested that women with a high BMI (≥ 25.0) have an associated increased miscarriage rate after ART treatment (Rittenberg et al., 2011a). A further study that examined the effect of BMI on miscarriage rate following ART using single blastocyst transfer demonstrated that women with a BMI between 25 and 35 are more than twice likely to have a clinical miscarriage compared to normal weight women, both in fresh and cryo-thawed cycles (Rittenberg et al., 2011b).

The pattern of early pregnancy loss in first trimester (EPL) provides an insight into the association with chromosomal abnormality of a miscarriage (Morikawa et al., 2004). With the availability of sensitive urine and serum b-hCG measurements in combination with high resolution trans-vaginal ultrasound, it is now possible to categorise early pregnancy losses into embryo loss (EL) – miscarriage of a pregnancy before a fetal heart is identified including empty gestational sac losses and fetal loss (FL) – miscarriage of a pregnancy once the fetal heart is seen on an ultrasound scan (Bricker and Farquharson, 2002), biochemical pregnancy loss - where the pregnancy was diagnosed by either serum or urine b-hCG without an ultrasound evaluation and the serial results

suggesting a pregnancy loss, pregnancy of unknown location - pregnancy loss not visualized on ultrasound with resolution of serum hCG and ectopic pregnancy – pregnancy outside the uterine cavity. The EPL pattern is not only essential for management, prognosis and follow-up of these women, particularly for women with recurrent miscarriage, but also provides an insight into an association with chromosomal abnormality of the implanting embryo.

My literature review in chapter 1, suggested that high BMI may be responsible for increased risk of spontaneous early pregnancy losses and a possible adverse effect of obesity on the peri-implantation endometrium.

The objectives of this study were [a] to describe the pregnancy loss pattern and its correlation to BMI in women who attended a tertiary implantation clinic with various reproductive failures and [b] to analyse whether the pregnancy loss pattern is significantly different between normal, overweight and obese women.

3.2 Materials and Methods

See chapter 2

3.3 Results:

3.3.1 Demographic details:

	All subjects (313)	Normal (n=164)	Overweight (n=97)	Obese (n=52)	p
Mean (± SD) Age	36.8 (4.7)	36.8(4.6)	37.0 (4.8)	36.3 (5.0)	0.69#
No. of EPL	969	432	326	211	-
Median (IQR) EPL	3.0 (0 – 5.0)	3.0 (0 – 4.0)	3.0 (1.0 – 5.0)	3.0 (2.0 – 6.7)	< 0.05##
No. of BPL	158	84	48	26	-
Median (IQR) BPL	0 (0 - 0)	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0.81##
No. of EGSL	525	219	172	134	-
Median (IQR) EGSL	0 (0 – 3.0)	0 (0 – 2.75)	0 (0 – 3.0)	1.0 (0 – 5.0)	< 0.05##
No. of FL	239	98	94	47	-
Median (IQR) FL	0 (0 – 1.0)	0 (0 – 1.0)	0 (0 – 1.0)	0 (0 – 1.75)	0.31##

Table 3.1: Maternal age and EPL patterns for all 313 subjects with reproductive failures included in the study. EPL: early pregnancy losses in the first trimester; BPL: biochemical pregnancy losses; EGSL: empty gestational sac losses; FL: fetal losses; SD: Standard deviation; IQR: inter-quartile range. $p < 0.05$ significant. # One way ANOVA, ##: Independent samples Kruskall-Wallis test.

A total of 313 women attended the clinic during the study period. The Kolmogorov-Smironov normality test with Lilliefors significance correction suggested that the data for age were normally distributed whereas the data for EPL, BPL, EGSL and FL were not normally distributed. The women were classified into three groups according to their BMI: normal (n=164), overweight (n=97) and obese (n=52). The demographic details and the clinical information for each of these groups are in Table 3.1. Out of the 969 first trimester miscarriages identified in these women, 158 (16.3%) were biochemical, 525 (54.18%) empty gestational sac losses and 239 (24.66%) were fetal losses. There was no significant difference in the mean (± SD) age in years for these women in different weight groups (p=.69).

3.3.2 Correlation of BMI and reproductive history:

Relationship between BMI and EPL, EGSL and FL indicated a significantly positive correlation of EPL (Spearman's correlation coefficient, rho .178, p=.002), EGSL (Spearman's correlation coefficient, rho .118, p=.037) and FL (Spearman's correlation coefficient, rho .130, p=.022) with BMI, whereas BPL was not significantly correlated with BMI (Spearman's correlation coefficient, rho -.027, p=.633) (Figure 3.2).

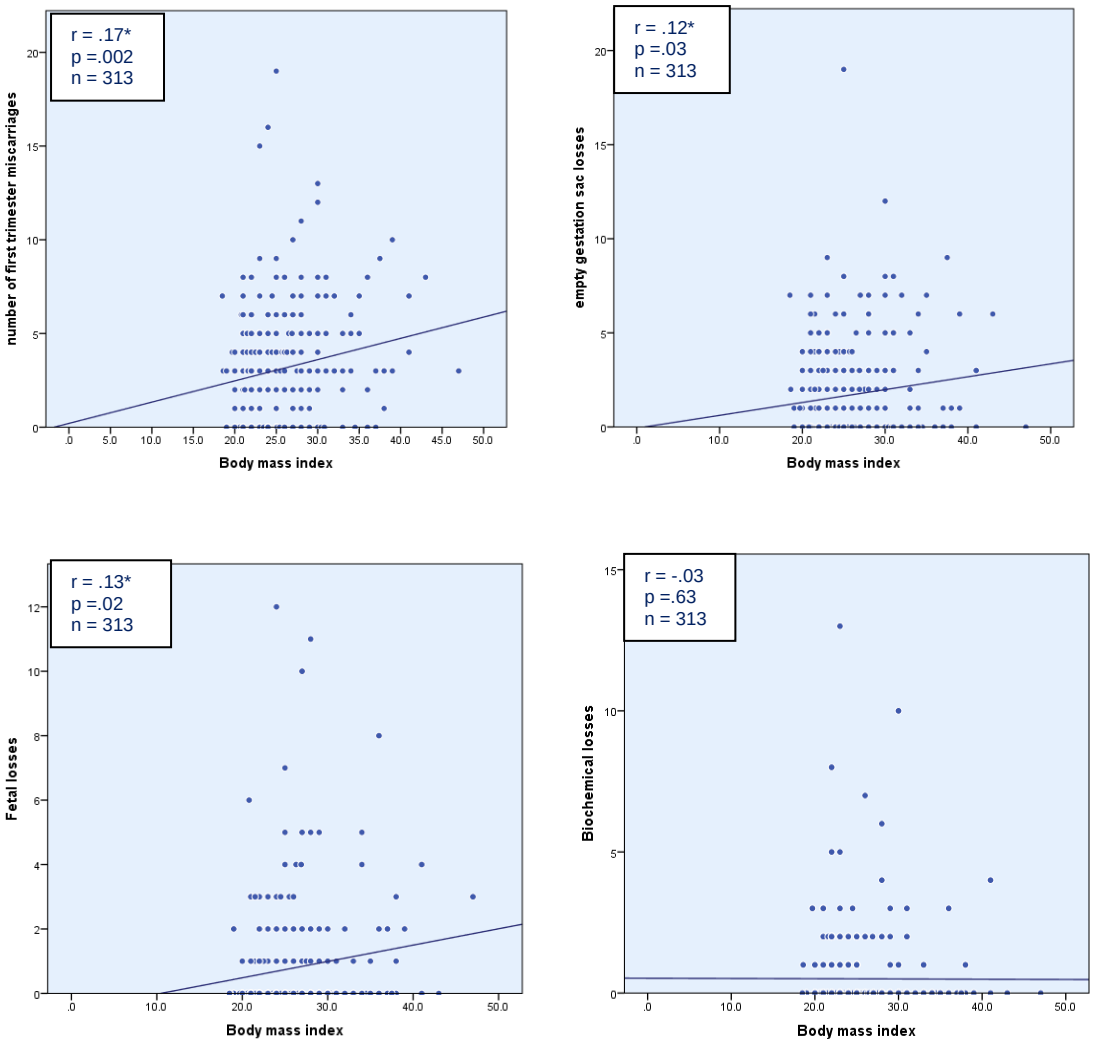


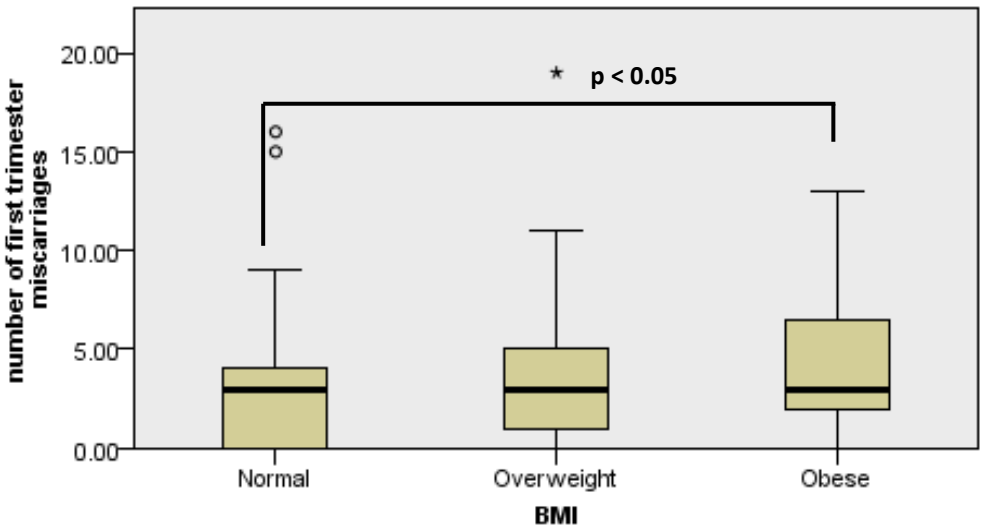
Figure 3.2: Relationship between BMI and EPL, EGSL and FL indicated a significantly positive correlation of EPL (Spearman's correlation coefficient, rho .178, p=.002), EGSL (Spearman's correlation coefficient, rho .118, p=.037) and FL (Spearman's correlation coefficient, rho .130, p=.022) with BMI, whereas BPL was not significantly correlated with BMI (Spearman's correlation coefficient, rho -.027, p=.633). EPL: early pregnancy losses in the first trimester; EGSL: empty gestational sac losses; FL: fetal losses; BPL: biochemical pregnancy losses.

3.3.4 Pregnancy loss pattern in BMI groups:

The Kruskal-Wallis test indicated a significant difference existed in the distribution of first trimester miscarriages among the three groups (T=10.007, df=2, p=.007) (Figure 3.3). A pair wise comparison was performed as a post-hoc follow-up which suggested a significant difference between the obese and normal weight women (p=.011), but there was no statistically significant difference between normal-overweight (p=.125) and overweight-obese (p=.706) groups.

Out of the 525 empty gestation sac pregnancy losses, 219 were in the normal weight women, 172 in overweight and 134 in obese women. The Kruskal-Wallis test indicated a significant difference in the distribution of EGSL existed among the three groups (T=7.658, df=2, p=.022) (Figure 3.4). A pair wise comparison suggested a significant difference between the obese and normal weight women (p=.018), but there was no statistically significant difference between the other groups (normal-overweight: p=.846; overweight-obese: p=.241)

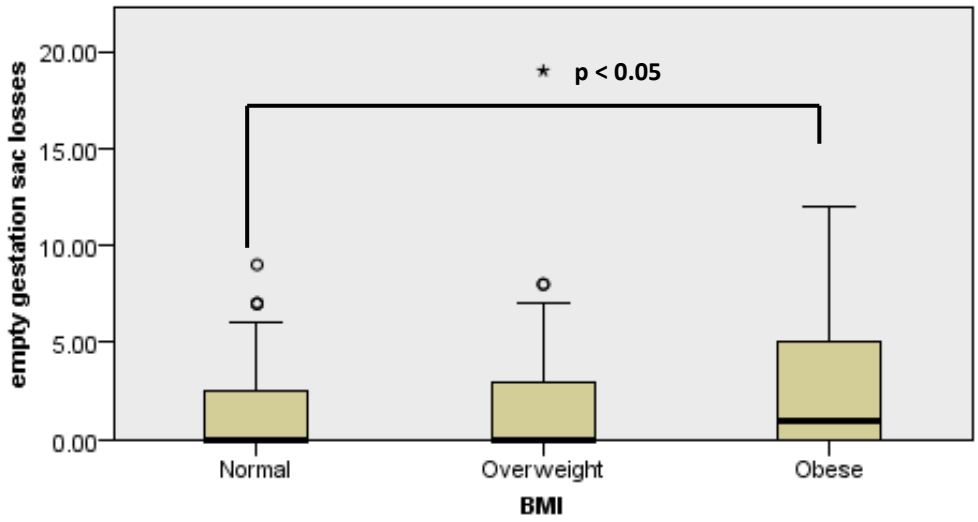
There was no statistically significant difference in the number of fetal losses (T=2.331, df=2, p=.312) and biochemical pregnancy miscarriages (T=0.406, df=2, p=.816) between three groups.



Each node shows the sample average rank of BMI.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Normal-Overweight	-23.259	11.427	-2.035	.042	.125
Normal-Obese	-41.450	14.198	-2.919	.004	.011
Overweight-Obese	-18.191	15.333	-1.186	.235	.706

Figure 2.3: The box and whisker plot showing the number of first trimester miscarriages in women with normal, overweight and obese women. The results of the Kruskal-Wallis test and pairwise comparisons for women with different weight groups are shown. A significant difference in the distribution of first trimester miscarriage was observed between obese and normal weight women.



Each node shows the sample average rank of BMI.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Normal-Overweight	-11.552	10.739	-1.076	.282	.846
Normal-Obese	-36.753	13.342	-2.755	.006	.018
Overweight-Obese	-25.201	14.409	-1.749	.080	.241

Figure 3.4: The box and whisker plot showing the number of empty gestation sac miscarriages in women with normal, overweight and obese women. The results of the Kruskal-Wallis test and pairwise comparisons for women with different weight groups are shown. A significant difference in the distribution of empty gestational sac losses was observed between obese and normal weight women.

3.4 Discussion

My study findings from a relatively large cohort of women, who attended a tertiary referral implantation clinic, support the evidence that obesity increases the risk of first trimester pregnancy loss. There is also a significant positive correlation between first trimester pregnancy losses and BMI.

From this study, I present a novel data of increased loss of empty gestational sac pregnancies or anembryonic miscarriages in obese women when compared to normal weight women. A significant positive correlation between the BMI and EGSL was also demonstrated.

Morikawa et al., (2004) examined miscarriage type and karyotype in RM women and found that embryo losses (included EGSL and embryonic miscarriages with embryo less than 3mm) are more likely to be karyotypically normal embryos whereas slightly advanced pregnancy losses (included FL with embryo more than 3mm and fetal miscarriages) are more likely to be pregnancies with abnormal karyotype. So the findings of our study may indicate that obese women have an increased risk of miscarriage of pregnancies which are less likely to have karyotypic abnormality i.e. due to oocyte abnormalities. This is in keeping with the findings of a study by Landres et al., (2010) which suggested that women with a BMI ≥ 25 are associated with an increased risk of miscarriage of embryos with a normal genetic component. A recent study by Boots et al., (2014) also proposed that obese women with RM have an increased risk of euploid miscarriage.

Endometrial dysfunction has been proposed to increase the risk of reproductive failures in obesity (Landres et al., 2010; Bellver et al., 2013) and our study findings appear to support this concept. It is likely that an impaired peri-implantation endometrial development associated with obesity predisposes to early implantation problems and therefore increases the risk of miscarriage of pregnancies with normal karyotype. Bellver et al., (2013) using first cycle ovum donation IVF/ICSI cycles with eggs obtained from normal weight donors, suggested that implantation, clinical pregnancy and live birth rates in recipients were significantly reduced with an increase in BMI.

The other possible explanation for an increased anembryonic miscarriage in obese women may be due to nutritional deficiencies associated with obesity. The EGSL can be considered to be a structural abnormality of pregnancy as there is no evidence of a fetus at the time of a miscarriage. The structural abnormalities are more common in obese women which are thought to be due to poor dietary habits of obese women who are often deficient in vitamins. Hence CMACE/RCOG joint guidelines (13) recommend the use of high dose folate and vitamin D supplements for all obese pregnant women.

Though in my study, the FL showed significantly positive correlation with BMI, there was no significant difference in the number FL between normal weight, overweight and obese subjects. A previous study which prospectively investigated 1200 ultrasound confirmed ongoing singleton intrauterine pregnancy at a mean gestational age of 9+ weeks did not find any significant difference in miscarriage rate in normal, overweight and obese women (Turner

et al., 2010). However, a recent study has suggested that in a first pregnancy, moderate and severe obesity increase the risk of fetal miscarriage following an ultrasound confirmation of fetal cardiac activity in the first trimester (O'Dwyer et al., 2012).

In my study, there was no significant difference in the number of biochemical pregnancy losses between obese and non-obese subjects. These are the spontaneously resolved pregnancies of unknown location and therefore could be anywhere in the upper reproductive tract (in fallopian tube, pelvis and on ovaries) or cervical canal and therefore not necessarily of endometrial aetiology.

The strength of my study is that it includes a relatively large sample size. My work has demonstrated an important association between early pregnancy loss patterns and BMI. I also report for the first time that superfertility is more prevalent in obese women presenting with recurrent miscarriage. We used the WHO classification of BMI to categorise women into different weight groups.

However, our study has several limitations and the main one relate to the retrospective data collection. The women at presentation to the clinic may not accurately remember the exact nature of first trimester pregnancy loss. The BMI at the time of an adverse pregnancy outcome may be different to BMI at presentation in the clinic which could have led to cross-over of women between the groups. Maternal age plays a vital role in predisposing women to a miscarriage. There was no difference in the age of women across the BMI

groups, but the age at presentation may not be a true reflection of the age at adverse outcome.

3.5 Conclusions:

By analysing a large number of women for their first trimester pregnancy loss pattern, my study supports the evidence that obesity may increase the risk of spontaneous first trimester pregnancy losses. In addition, the findings of an increased anembryonic miscarriage in obese women suggests of a possible increased loss of euploid embryos as a result of an endometrial dysfunction in the peri-implantation period. The findings of this study may assist in designing large, well-designed, prospective studies which are essential to establish the effects of BMI on first trimester pregnancy loss patterns.

CHAPTER 4:

THE EFFECT OF OBESITY ON TIME TO PREGNANCY INTERVAL AND EARLY PREGNANCY LOSS PATTERN IN WOMEN WITH RECURRENT MISCARRIAGE

4.1 Introduction

Loss of three or more consecutive pregnancies before 24 weeks of gestation is the most acceptable definition for recurrent miscarriage (RM) (Stirrat 1990) in Europe, but has recently been defined by the American Society for Reproductive Medicine as two or more failed pregnancies (ASRM, 2008).

The time taken to achieve pregnancy (TTP) expressed as monthly fecundity rates (MFR), i.e. the probability of achieving a pregnancy within one menstrual cycle (Evers, 2002) can be used to measure the fertility rates. The average MFR for couples is relatively low at 20% (Leridon and Spira, 1984) and high prevalence of aneuploid human embryos may be responsible for this relative reproductive inefficiency in humans. Based on the MFR, it is predicted from a simple hypothetical model that 74, 93 and 100% of normal fertile couples will conceive within 6, 12 and 24 months, respectively (Evers, 2002). One in six couples in developed countries are affected with subfertility and infertility and these couples have a reproductive inefficacy with MFR of 1-5% and 0%, respectively (Evers, 2002). Whereas, 3% of the couple have superfertility with a MFR of 60% or more and can achieve 94% and 100% of pregnancies within 3 and 6 months, respectively (Evers, 2002). It is also predicted that the proportion of women achieving 3 or more consecutive pregnancies within 1, 3 and 6 months to be 0.8%, 8% and 41% respectively (Salker et al., 2010). Superfertility in RM women is more than the average expected for general population and these women with RM report a very short TTP (Salker et al., 2010). This supports the concept that an increase MFR in RM women is likely to be a result

of a prolonged implantation window secondary to an impaired endometrial decidualisation, which allows even the developmentally compromised embryos to implant which subsequently miscarry.

We hypothesised that obese women with recurrent miscarriage have a significantly shorter time to pregnancy and an increased first trimester pregnancy losses with no difference in the pregnancy loss pattern when compared to normal weight women with recurrent miscarriage.

4.2 Subjects and Methods

See chapter 2

4.3 Results

4.3.1 Demographic details:

A total of 177 women who attended the clinic during the study period were identified to have had a history of RM. The demographic details and the clinical information for each of these groups are in Table 4.1. The data for age was normally distributed, whereas the data for EPL, EGSL, FL and BPL were not normally distributed.

	All subjects (n=177)	Normal (n=84)	Overweight (n=57)	Obese (n=36)	p
Mean (± SD) Age	36.9 (4.5)	36.5 (4.3)	37.2 (4.7)	37.3 (4.3)	0.60#
No. of EPL	879	383	294	202	-
Median (IQR) EPL	4 (3 – 6)	4 (3 – 5)	4 (3.5 – 6)	5 (3 – 7)	<0.05##
No. of BPL	129	66	42	21	-
Median (IQR) BPL	0 (0 - 0)	0 (0 – 1)	0 (0 – 1)	0 (0 – 0)	0.39##
No. of EGSL	495	204	160	130	-
Median (IQR) EGSL	3 (0 – 4)	2 (0 – 4)	2 (0 – 4)	3 (1 – 6)	0.18##
No. of FL	211	84	83	44	-
Median (IQR) FL	0 (0 – 2)	0 (0 – 2)	0 (0 – 2)	0 (0 – 2)	0.79##

Table 4.1: Maternal age and EPL patterns for all 177 subjects with recurrent miscarriage. EPL: early pregnancy losses in the first trimester; BPL: biochemical pregnancy losses; EGSL: empty gestational sac losses; FL: fetal losses; SD: Standard deviation; IQR: inter-quartile range. $p < 0.05$ significant. # One way ANOVA, ##: Independent samples Kruskall-Wallis test.

4.3.2 Correlation of BMI and pregnancy loss pattern in women with recurrent miscarriage

Relationship between BMI and EPL indicated a significantly positive correlation of EPL (Spearman's correlation coefficient, rho .160, p=.033) with BMI, whereas EGSL (Spearman's correlation coefficient, rho .077, p=.311) and FL (Spearman's correlation coefficient, rho .082, p=.275) BPL (Spearman's correlation coefficient, rho -.068, p=.369) were not significantly correlated with BMI (Figure 4.1).

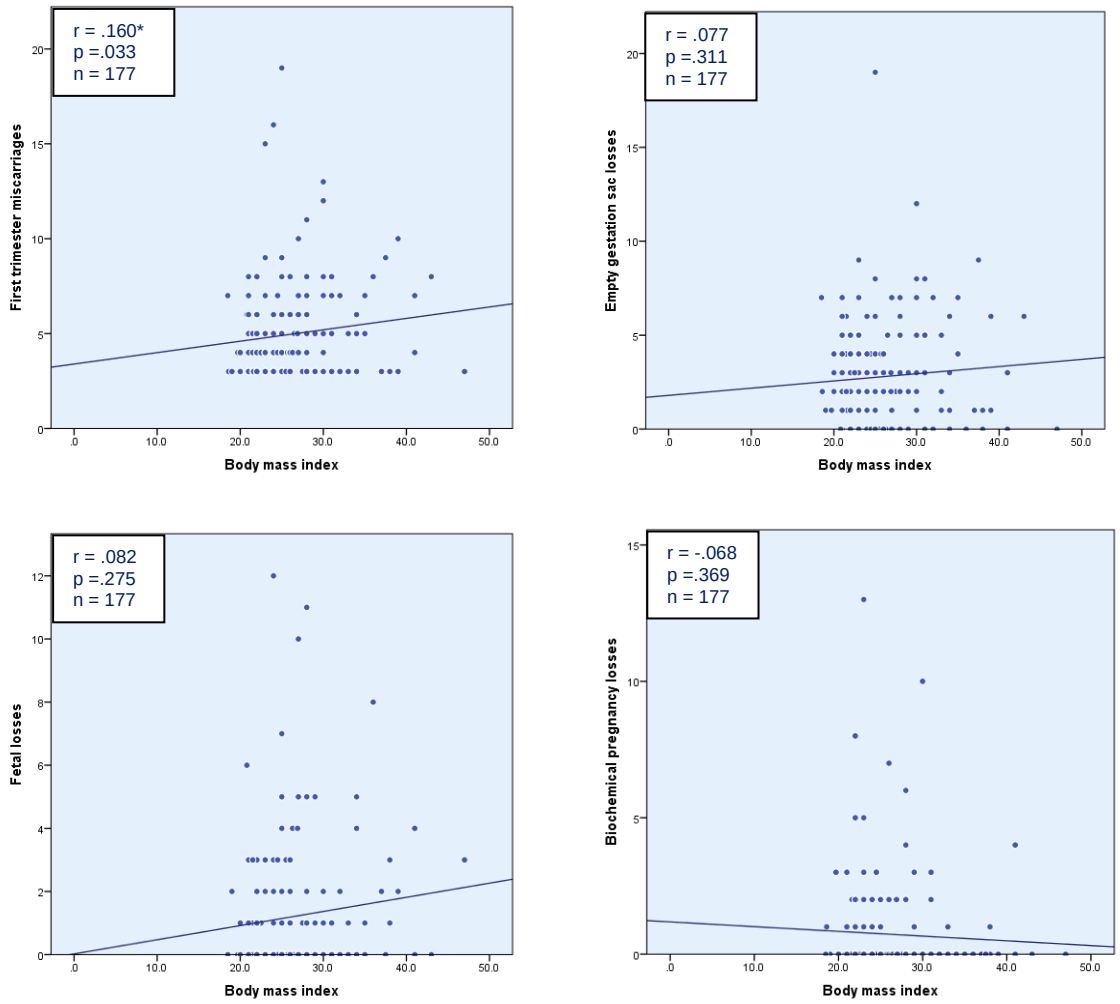
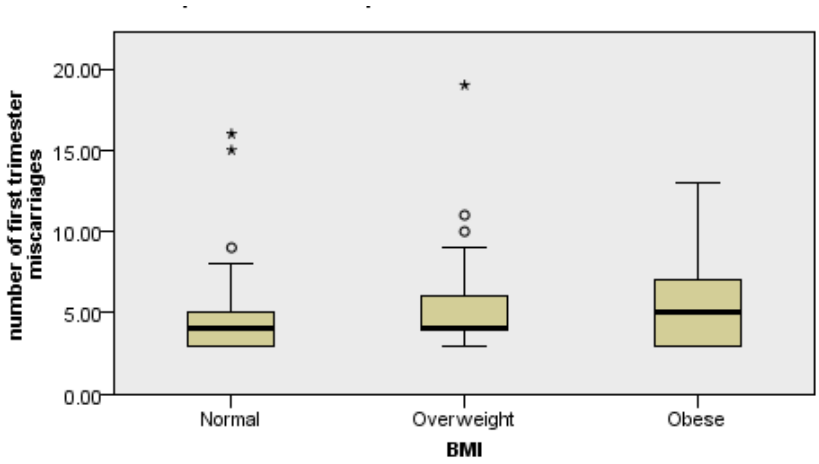


Figure 4.1: Relationship between BMI and EPL indicated a significantly positive correlation of EPL (Spearman's correlation coefficient, rho .160, p=.033) with BMI, whereas EGSL (Spearman's correlation coefficient, rho .077, p=.311) and FL (Spearman's correlation coefficient, rho .082, p=.275) BPL (Spearman's correlation coefficient, rho -.068, p=.369) were not significantly correlated with BMI. EPL: early pregnancy losses in the first trimester; EGSL: empty gestational sac losses; FL: fetal losses; BPL: biochemical pregnancy losses.

4.3.3 Pregnancy loss pattern of women with recurrent miscarriage in different body mass index groups

The Kruskal-Wallis test indicated a significant difference existed in the distribution of first trimester miscarriages among the three groups (T=6.047, df=2, p=.049) (Figure 4.2). A pair-wise comparison did not identify any statistically significant difference between the obese and normal weight women (p=.079), normal-overweight (p=.250) and overweight-obese (p=1.00) groups.



Each node shows the sample average rank of BMI.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Normal-Overweight	-14.789	8.565	-1.727	.084	.253
Normal-Obese	-22.298	9.942	-2.243	.025	.075
Overweight-Obese	-7.509	10.625	-.707	.480	1.000

Figure 4.2: The box and whisker plot showing the number of first trimester miscarriages in women with normal, overweight and obese women with a history of RMC. The results of the Kruskal-Wallis test and pairwise comparisons for women with different weight groups are shown.

There was no statistically significant difference in the number of empty gestational sac losses (T=3.400, df=2, p=.183), fetal losses (T=.471, df=2, p=.790) and biochemical pregnancy miscarriages (T=1.884, df=2, p=.390) between the three groups (not shown).

4.3.4 Time to pregnancy intervals

The data for RM, BMI and time to pregnancy intervals was available for a total of 154 women. The women were classified according to their BMI into normal (n=74), overweight (n=47) and obese (n=33) groups (Table 4.2).

Overall, 33% conceived within I month, 55% within first three months and 69% within the first six months, significantly quicker than that predicted in a normal fertile population (0.8%, 8% and 41% respectively, adapted from Salker et al., 2010). Obese women had cumulative pregnancy rates of 71.4% and 85.7% by 3 and 6 months respectively which was higher than the cumulative pregnancy rates for normal weight (48.4% and 64%) and overweight (52.6% and 63.1%) groups (Table 4.3).

BMI (Binned)	Total number	Number of Events	Censored	
			N	Percent
Normal	74	74	0	0.0%
Over weight	47	47	0	0.0%
Obese	33	33	0	0.0%
Overall	154	154	0	0.0%

Table 4.2: Number of women with recurrent miscarriage and time to pregnancy intervals

	1 Month	3 Months	6 Months
Predicted	0.8%	8%	41%
Overall (n=154)	33%	55%	69%
Normal BMI (n=74)	32.8%	48.4%	64%
Overweight (n=47)	28.9%	52.6%	63.1%
Obese (n=33)	39.2%	71.4%	85.7%

Table 4.3: Summary of ≥ 3 cumulative pregnancy rates predicted at 1 month, 3 months and 6 months for the general population and women included in this study. The women were grouped into normal, overweight and obese according to their BMI.

Comparison of survival curves using Kaplan–Meier statistics (Mantel-Cox log rank test) indicated a significant difference ($p < 0.05$) in the mean time in months to conceive at 3 months for obese women with RM when compared to normal and overweight women with RM (Figure 4.3).

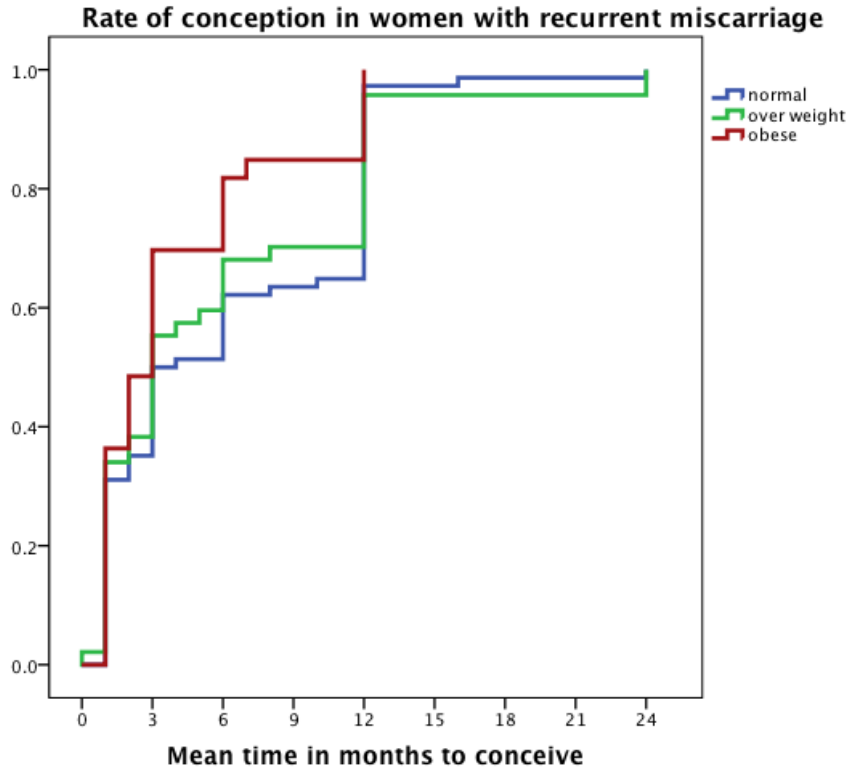


Figure 4.3: Kaplan-Meier survival curves for TTP intervals for normal, overweight and obese women. The overall comparison using log rank (Montel-Cox) indicated a statistically significant difference amongst the groups with more obese women with RM had a shorter TTP when compared to normal and overweight women.

4.4 Discussion

My study findings, from a relatively large cohort of women with a history of RM, support the evidence that increased female BMI has a negative influence on the early pregnancy outcome.

In these women who attended our tertiary referral implantation clinic, I found a significant positive correlation between the BMI and first trimester pregnancy losses (EPL), but no significant correlation between BMI and EGSL, FL and BPL. My study also found a statistically significant difference in the first trimester miscarriages in different weight groups classified according to their BMI, but failed to identify significant difference in the EGSL, FL and BPL.

A previous study that examined miscarriage type and fetal karyotypes in RM women found that, embryo losses (EGSL) are more likely to be karyotypically normal embryos and FL to be aneuploid embryos (Morikawa et al., 2004). So the findings of our study indicate that in women with a history of RM, obesity increases the risk of first trimester miscarriages, but it does not affect the type of miscarriage and hence likely the karyotype of the conceptus. The BMI has no significant effect on the miscarriages of both euploid and aneuploid pregnancies in women with RM. This finding is in contrary to the hypothesis by Salker et al., (2010) which proposed that RM could primarily reflect poor embryo selection, accounting for an increased prevalence of aneuploidic miscarriages and to the findings of Sugiura-Ogasawara et al., (2012) which found that aneuploidy was

the most common cause of RM and of Boots et al., (2014) which suggested that obese women with RM have an increased frequency of euploid miscarriage.

The other findings of my study support the concept of 'biological superfertility' within a RM population and of a novel finding that superfertility is more prevalent amongst obese than in normal weight women with a history of RM.

The overall ≥ 3 cumulative pregnancy rates in our study group was 55% and 69% by 3 months and 6 months, respectively, which is significantly quicker than that predicted for a normal fertile population (8% and 41% respectively). These findings are consistent with the findings of a study by Salker et al., (2010) who demonstrated that women with RM reported a considerably shorter time to pregnancy. In their retrospective analysis of TTP from women attending a tertiary service level RMC clinic, 41% of the women with history of RMC had a time to pregnancy interval of 3 months or less compared to 8% as predicted for general population. A recent study by Orlando and Coulam (2014) also identified that a 32% of the women with RM in their cohort are superfertile.

From my study population, I report a novel finding of significantly more obese women achieved pregnancy within 3 months (71.4%) when compared to normal weight (48.4%) and overweight (52.6%) women.

My work has highlighted important connections between BMI, RM and superfertility. There is a good body of evidence, from in-vitro studies, to suggest that inability of the peri-implantation endometrium to mount an adequate

decidual response impairs embryo recognition and selection upon implantation (Salker et al., 2010; Salker et al., 2011; Salker et al., 2012; Weimer et al., 2012), which leads to shorter TTP intervals and predispose to RM (Salker et al., 2010). This abnormal phenotype results in extended window of implantation which reduces the endometrial ability to be 'selective' in response to embryo quality (Teklenburg et al., 2010a). This concept is consistent with the previously reported association of late implantation of the embryos with an increased risk of early miscarriage (Wilcox et al., 1999).

My findings suggest that obesogenic environment may have a negative influence on the peri-implantation endometrium which results in an increased prevalence of 'biological superfertility' in obese women when compared to normal and overweight women, which in turn predisposes them to RM. This impaired endometrial transformation with an extended window of implantation allows the embryos, both developmentally competent as well as compromised, to implant, but finally results in a clinical EPL as the endometrium fails to support the highly orchestrated implantation. The pregnancies that are lost as a result of superfertility could either be euploid or aneuploid, which explains the finding of no different EGSL and FL between different weight groups.

The strength of my study is that it includes a relatively large sample size. My work has demonstrated an important association between early pregnancy loss patterns and BMI. I also report for the first time that superfertility is more prevalent in obese women presenting with recurrent miscarriage. I used the WHO classification of BMI to categorise women into different weight groups.

However, the study has several limitations which relate to data collection. The women at presentation to the clinic may not accurately remember the exact nature of EPL and the exact time required to achieve each pregnancy. The BMI at the time of an adverse pregnancy outcome may be different to BMI at presentation to the clinic which could have led to cross-over of women between the groups. Maternal age plays a vital role in predisposing women to a miscarriage. Conception and successful pregnancy are dependent on many variables and maternal age plays a vital role in predisposing women to miscarriages. Though there was no significant difference in the age of women across the BMI groups, the age at presentation to the clinic may not be a true reflection of the age at the time of adverse outcome.

4.5 Conclusions

The findings of my study support the evidence that the metabolic effects of obesity may cause reproductive failure by its harmful effects on the endometrium, which impairs the fine balance between receptivity and embryo selection. Further research to describe the peri-implantation endometrium and to study the exact mechanism by which the obesity affects endometrium is required. If confirmed, clinical trials with therapeutic targets to improve decidualisation and thus reproductive outcome in obese women could hold significant clinical potential.

CHAPTER 5:

DOES OBESITY AFFECT UTERINE LEUCOCYTE POPULATION?

5.1 Introduction:

Obesity represents a state of chronic subclinical inflammation. Hypertrophy of the adipocytes leads to hypoperfusion and hypoxia and endoplasmic reticulum (ER) stress are some of the mechanisms by which obesity results in increased adipose tissue inflammation. (Bluher M. 2009). Increased release of fatty acids, hormones, and pro-inflammatory molecules occur in obesity which is due to altered adipose tissue metabolic and endocrine function. Reactive peripheral leukocytosis and elevated acute phase reactants are found to be associated with obesity. (Herishanu et al., 2006)

As discussed in chpater 1, there has been a growing interest in endometrial leucocyte population among women with poor reproductive outcome (Quenby et al., 1999; Quenby and Farquharson, 2006). Obese pregnant women are found to have an increased systemic and placental inflammation and associated endocrine and immune functions (Stewart et al., 2007; Challier et al., 2008). Defective decidualisation is associated with women with a high uNK cell density of more than five percent in the peri-implantation endometrium (Kuroda et al., 2013). Women with RMC have increased uNK cell density in the peri-implantation endometrium (Lachapelle *et al*., 1996; Quenby *et al*., 1999, 2005, 2009; Tuckerman *et al*., 2007).

We hypothesised that the leucocytes density in the peri-implantation endometrium, the immune cells which are likely to play a role in reproductive

success, is significantly different in obese women when compared to that of normal weight women.

5.2 Materials and Methods

As described in chapter 2

5.3 Results

5.3.1 Uterine natural killer cells

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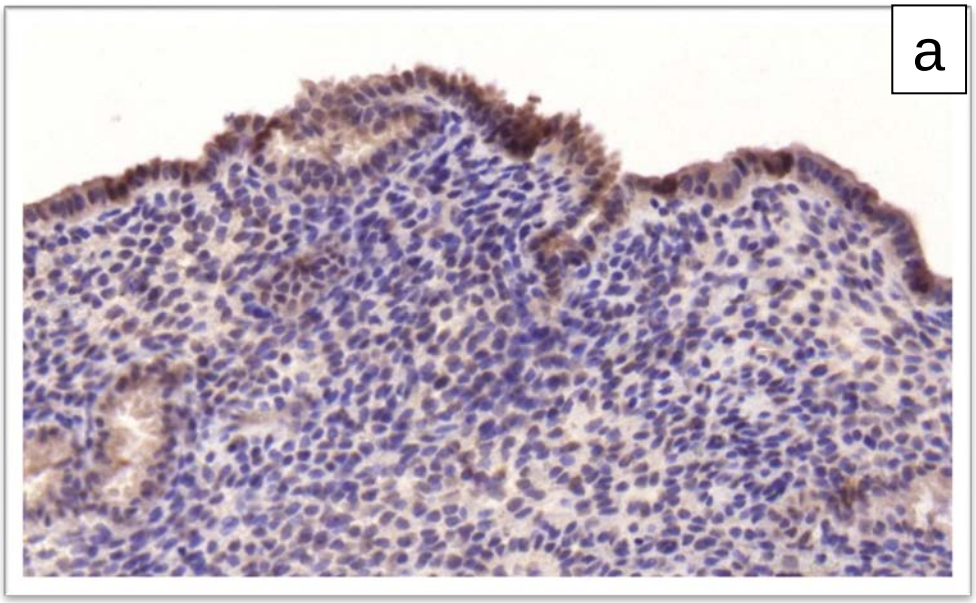


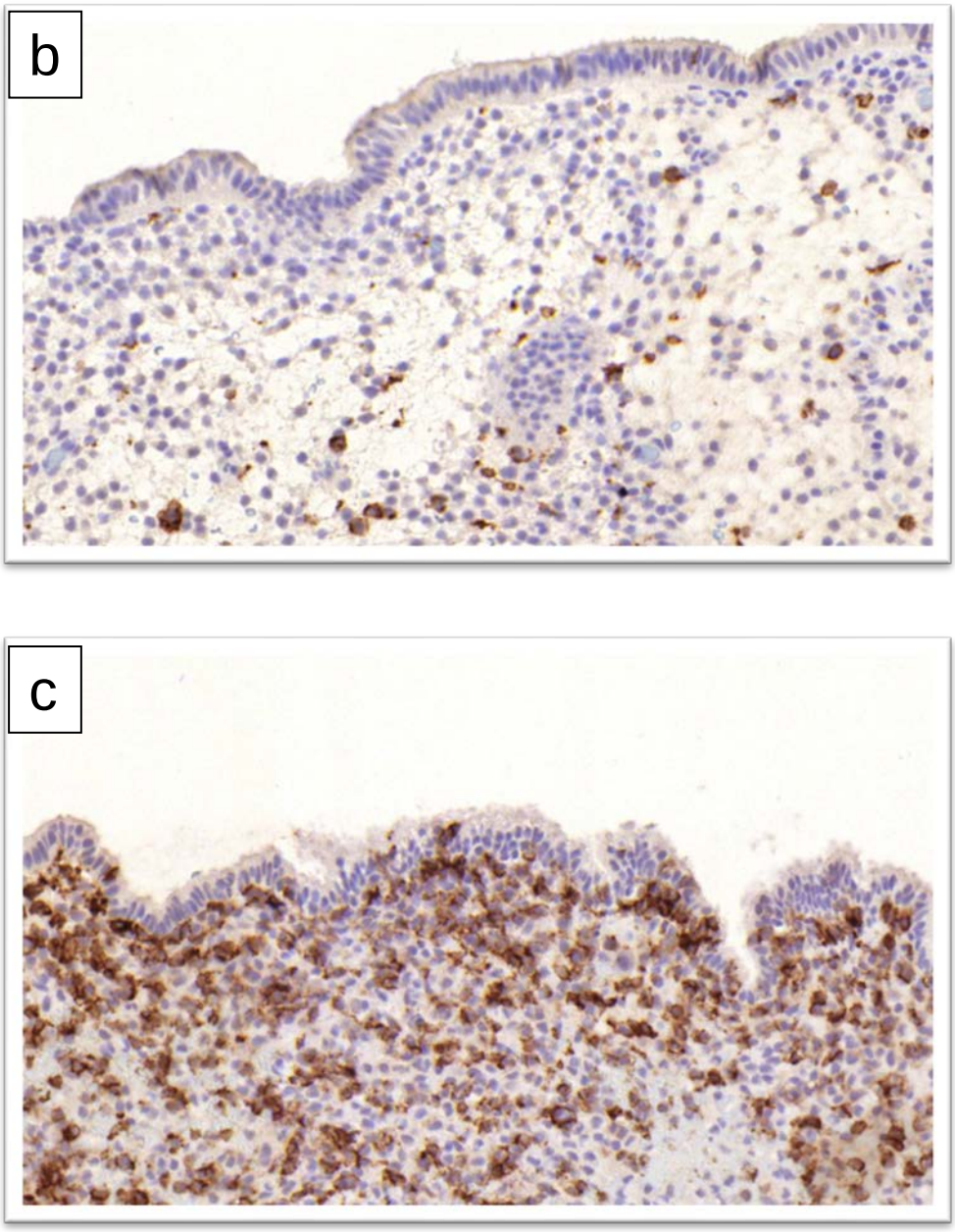


Figure 5.1: Immunohistochemical staining of $CD56^{+}$ cells in the human peri-implantation endometrium. a) Negative control stained with mouse IgG; b) subject with < 5% of the uNK cell density; c) high (>5%) uNK cell density. 400 x Magnification

5.3.1.1 Demographic details:

The endometrial biopsies were obtained from a total of 319 women, but the uNK cell density was available for 304 women. The others were not suitable for uNK

cell analysis. Tests for normality suggested that age was the only variable that was normally distributed, whereas uNK cell density, BMI, number of livebirths, first trimester miscarriages, number of biochemical losses, number of fetal losses, number of empty gestational sac losses did not pass the normality test. These women were categorized into three groups according to their BMI – Normal BMI (n=162), Overweight (n=92) and Obese (n=50). The demographic details are in Table 5.1).

	Normal (n=162)	**Overweight (n=92)**	**Obese (n=50)**	**p**
Age in years – Mean (± SD)	40.73 (± 4.67)	41.09 (± 4.77)	40.33 (± 4.86)	.501#
BMI (Kg/m^2) - Median (IQR)	22 (21 - 23)	26.7 (25 - 28)	33.0 (30 - 36)	<.0001***##
Women with previous Live Birth (%)	34 (20.98)	22 (23.91)	11 (22)	.864###
Recurrent Miscarriage (%)	84 (51.85)	53 (57.60)	34 (68.84)	.125###

Table 5.1: Summary of clinical details of the study population who were assessed for peri-implantation endometrial uterine natural killer cell density. #: One-way ANOVA, ##: Kruskall-Wallis non parametric test, ###: Chi square test, *** statistically significant difference.

5.3.3.2 Correlation of BMI and uNK cell density:

BMI was not significantly correlated with uNK cell density. Spearman's rank correlation coefficient (rho) was -.035 with two-tailed p=.538. (Figure 5.2)

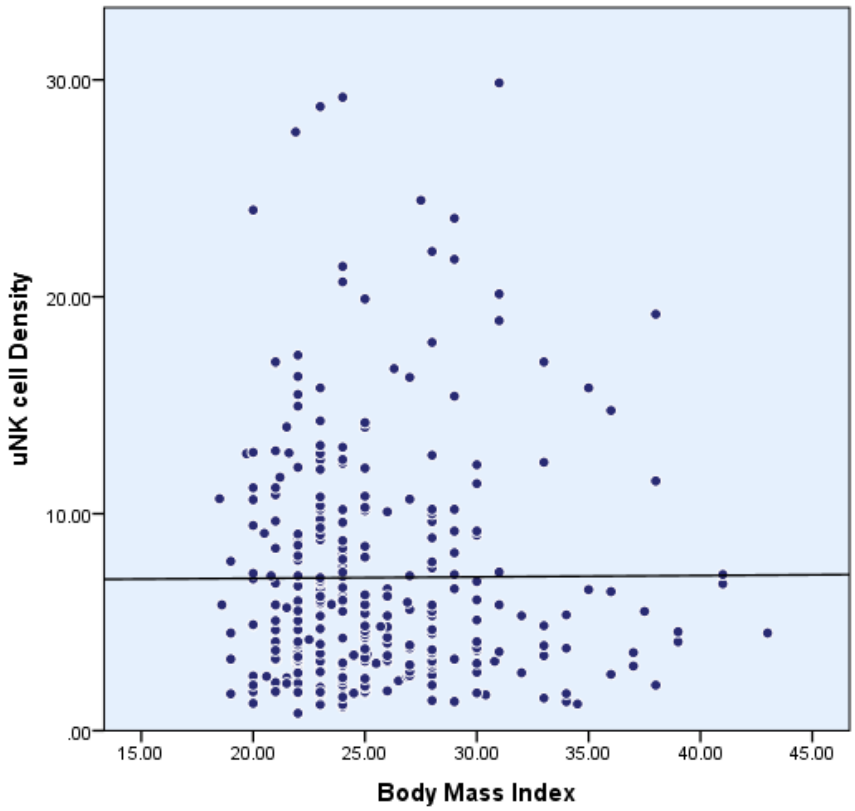


Figure 5.2: Scatter plot of correlation of uNK cell density and BMI (n=304). No significant correlation was observed (Spearman's rho = -.035, p=.538)

5.3.3.3 uNK cell density in BMI groups:

The median (IQR) uNK cell density in the normal weight group was 5.85 (3.19 – 9.61) compared to 4.81 (3.20 – 9.53) in the overweight group and 4.97 (3.17 –

9.05) in obese group (Figure 5.3). An independent samples Kruskal-Wallis test indicated no significant difference in the distribution of uNK cells among the three groups (T=.576, df=2, p=.758).

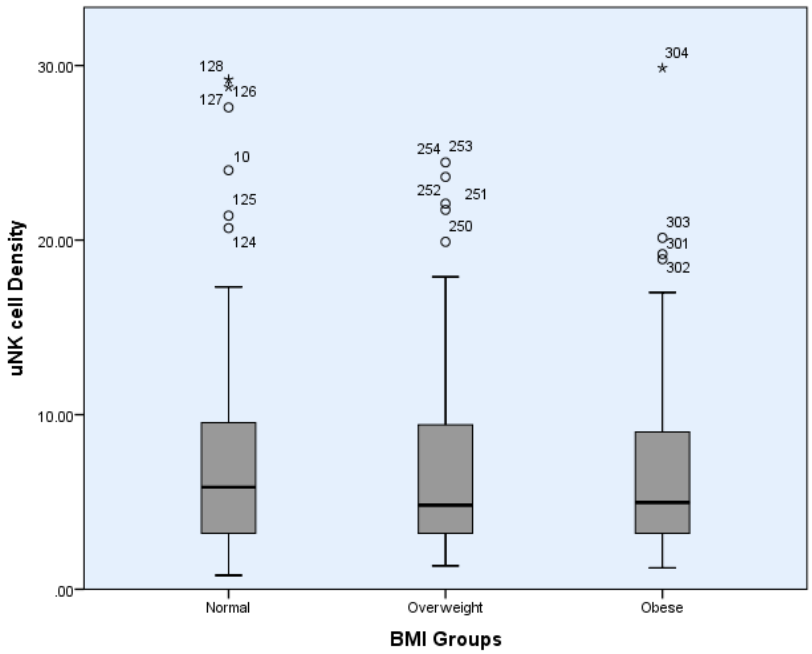


Figure 5.3: Box and whisker plot showing the uNK cell density in the peri-implantation endometrium from women of different BMI groups. No significant difference in the distribution of uNK cell density in different weight groups. Kruskal-Wallis nonparametric test, p=.758

5.3.3.4 uNK cell density and age

The age and uNK cell density was available from 299 women. The distribution of uNK cell density was not normally distributed in different age groups. Age was not significantly correlated with uNK cell density. Spearman's rank correlation coefficient (rho) was .041 with two-tailed p=.484. The women were grouped according to their age, (i) Age less than 35 years (n=105), (ii) Age between 35 - 39.9 years (n=122) and (iii) Age ≥ 40 years (n=72). An

independent samples Kruskal-Wallis test indicated no significant difference in the distribution of uNK cells among the three groups (T=.923, df=2, p=.630) (Figure 5.4).

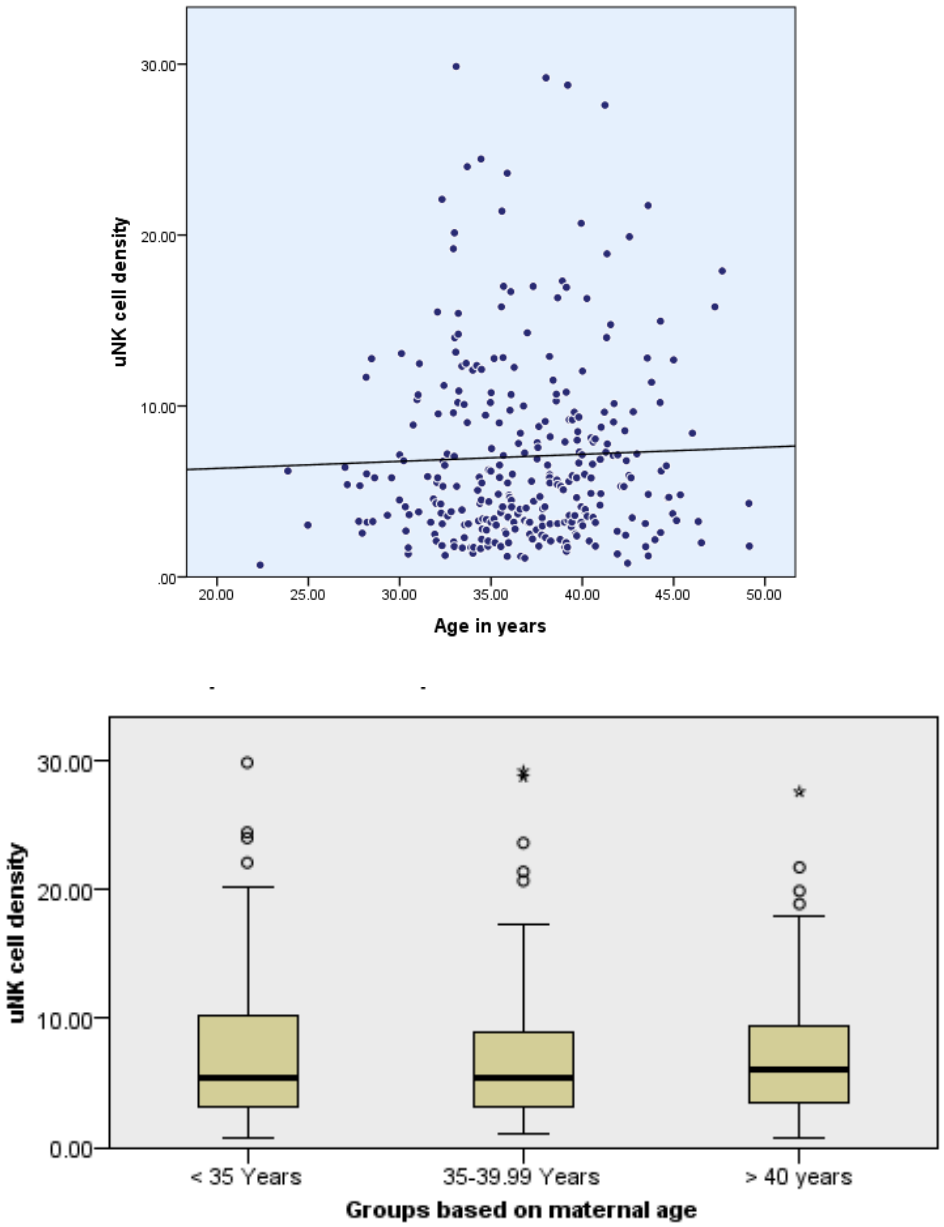


Figure 5.4: Scatter plot of correlation of uNK cell density and age (n=299). No significant correlation was observed (Spearman's rho = .041, p=.484). Box and whisker plot showing the uNK cell density in different age groups.

5.3.3.5 The effect of BMI on uNK cell density in women with RM

There were 171 women with RMC and they were categorized into three groups according to their BMI at presentation – normal (n=84), overweight (53) and obese (n=34). There was no significant correlation between the uNK cell density in the peri-implantation endometrium and BMI in women with a history of RMC (Spearman's rho, correlation coefficient = .095, p.219) (Figure 5.5).

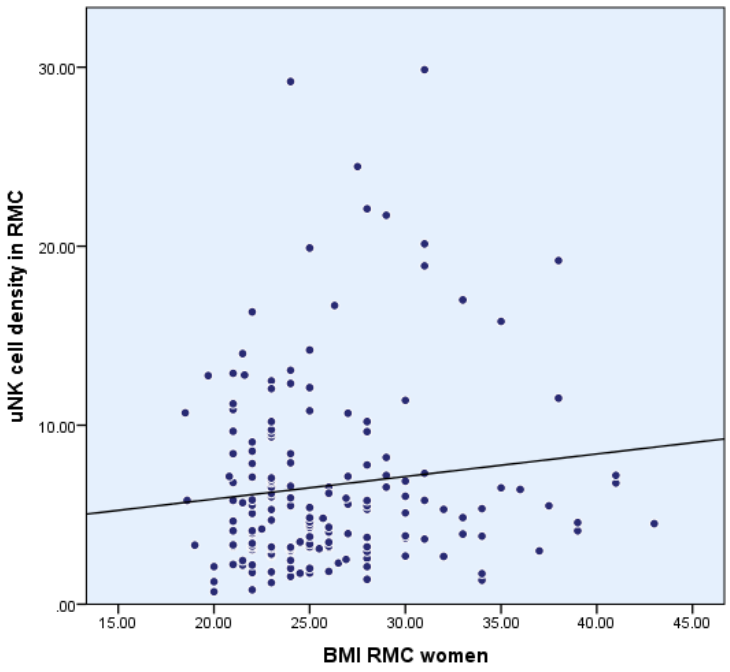


Figure 5.5: Scatter plot showing correlation of BMI and uNK cell density in women with RMC. No significant correlation was observed (Spearman's rho = .095, p=.219)

The median (IQR) uNK cell density in the normal weight women with RMC was 5.18 (3.00 – 8.41) compared to 4.80 (3.20 – 7.99) in overweight women with RMC and 5.42 (3.81 – 8.33). An independent samples Kruskal-Wallis test indicated no significant difference in the distribution of uNK cells existed among the three groups (T=1.826, df=2, p.401) (Figure 5.6).

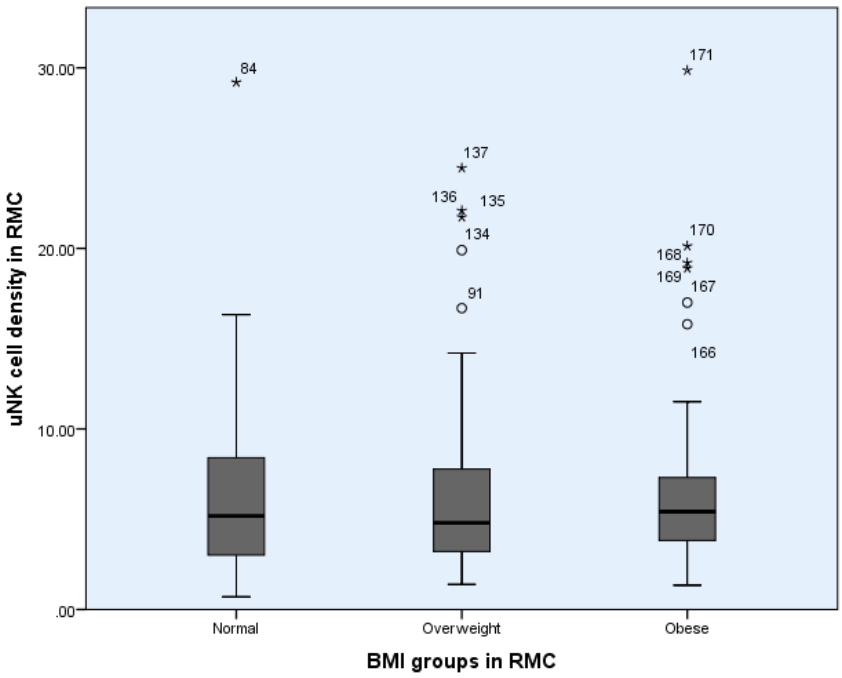
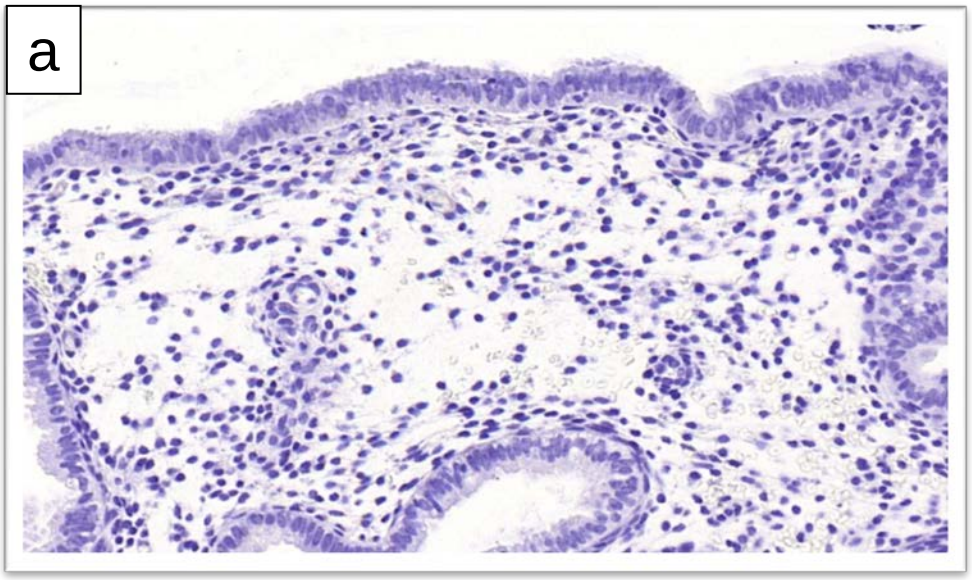
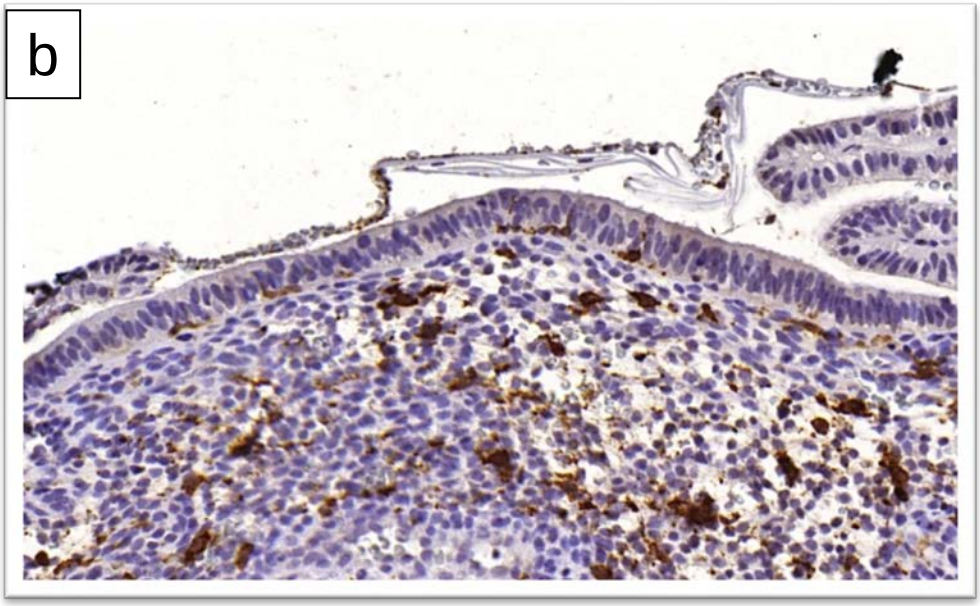


Figure 5.6: Box and whisker plot showing the uNK cell density in the peri-implantation endometrium from RMC women of different BMI groups. No significant difference in the distribution of uNK cell density in different weight groups of women with RMC. Kruskal-Wallis nonparametric test, p=.401)

5.3.2 Macrophages





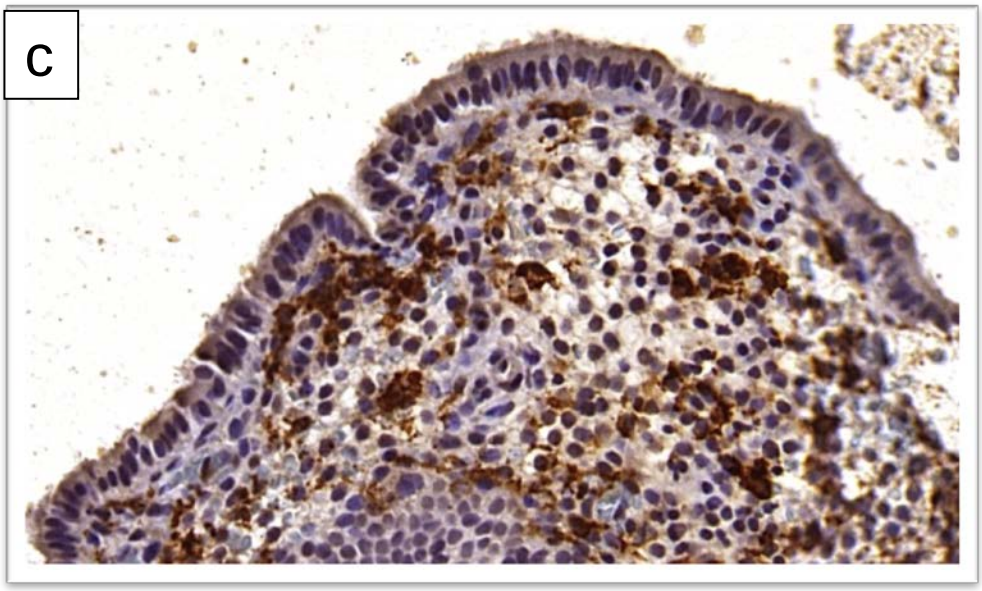


Figure 5.7: Immunohistochemical staining of CD14+ cells in the human peri-implantation endometrium. a) Negative control stained with mouse IgG; Sub-epithelial region of the endometrium with stromal cells stained in purple with b) low density and c) high density macrophages in brown. 400 x Magnification.

5.3.2.1 Demographic details:

The endometrial biopsies were obtained from 20 women (Table 5.2) and they were categorized into two groups according to their BMI – Normal BMI (n=10) and Obese (n=10). The age and BMI were normally distributed, whereas the first trimester miscarriages and macrophage density did not pass the normality test. There was no statistically significant difference in the mean (± SD) age in years between these two groups. The distribution of first trimester miscarriages was significantly higher in obese women compared to normal weight women (Table 5.3).

Age in years	Hospital Number	No of first trimester miscarriages	BMI	Macrophage density (%)
42	AA1435715	3	23	10.75
38	AA1451290	4	21	5.92
41	AA1452040	4	21.5	3.7
31	AA1492850	2	20	4.73
36	AA1456186	0	24	0.89
39	AA1461100	3	18	9.22
35	AA1475799	0	23.5	4.35
33	AA1484437	3	22	3.74
33	AA1493198	3	21	3.86
37	AA1495478	6	21	5.79
38	AA1437164	4	30	4.03
31	AA1467510	0	30.8	3.08
35	AA1454032	9	37.5	5.08
32	AA1462077	7	41	9.89
29	AA1406130	8	43	5.28
27	AA1480451	8	36	9.25
33	AA1482020	5	31	4.95
27	AA1488932	6	34	2.79
41	AA1491000	3	32	4.47
35	AA1495502	7	35	5.06

Table 5.2: Individual clinical data of women assessed for macrophage density

	Normal (n=10)	Obese (n=10)	p
Age in years – Mean (± SD)	36.85 (3.61)	33.44 (4.73)	.417[#]
BMI (Kg/m^2) - Mean (± SD)	21.5 (1.76)	35.03 (4.42)	<.0001[***#]
Median (± IQR) First Trimester Miscarriages	3.0 (1.5 – 4.0)	6.5 (3.75 – 8.0)	< 0.05[##]
Median (± IQR) $CD14^+$ density	4.54 (3.73 – 6.74)	5.01 (3.79 – 6.27)	.853[##]

Table 5.3: Summary of clinical details of the study population for peri-implantation endometrial macrophage density, #: Independent samples test, ##: Independent samples Mann-Whitney U test.

5.3.2.2 Correlation of BMI and macrophage density:

BMI was not significantly correlated with peri-implantation endometrial macrophage density. Spearman's rank correlation coefficient (rho) was .099 with two-tailed $p > 0.05$ for macrophage density and BMI (Figure 5.8).

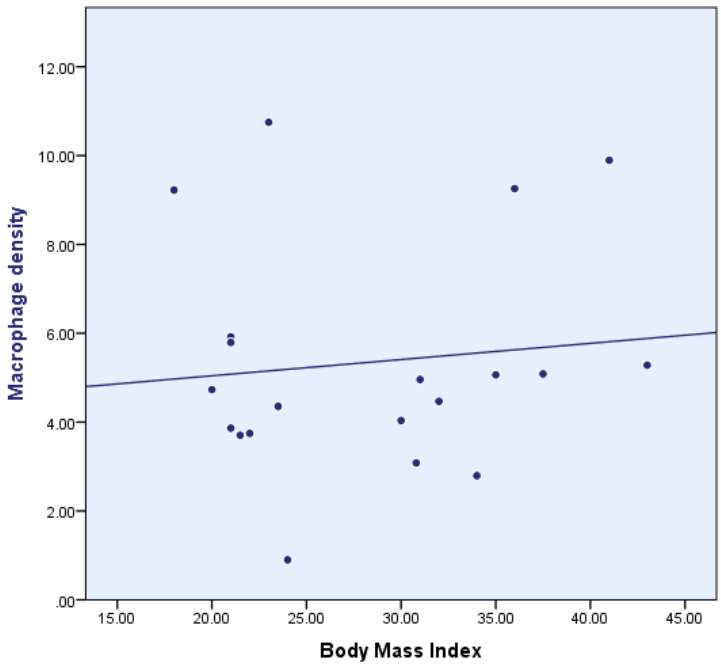


Figure 5.8: Scatter plot of correlation of macrophage density with BMI (n=20). No significant correlation was observed between macrophage and BMI (Spearman's rho = .099, p=.677)

5.3.2.3 Macrophage density in BMI groups:

The median (IQR) uNK cell density in the normal weight group was 4.54 (3.73 – 6.74) when compared to 5.01 (3.79 – 6.27). An independent samples Mann-Whitney test indicated no significant difference in the distribution of macrophages existed among the two groups (T=.227, p.853) (Figure 5.9).

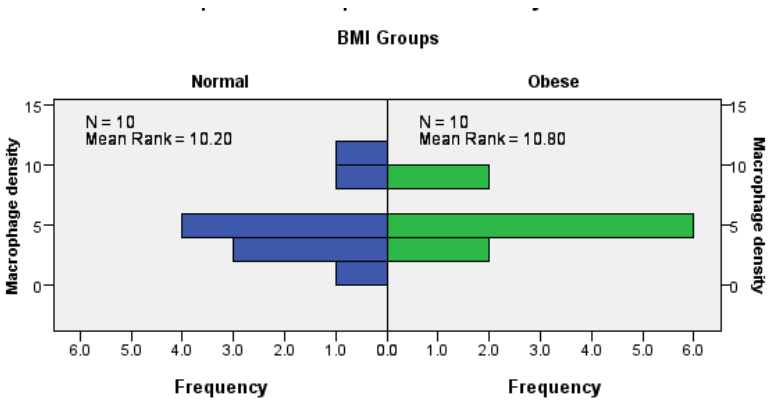


Figure 5.9: Independent samples Mann-Whitney U test showing the macrophage density in the peri-implantation endometrium from women of different BMI groups. No significant difference in the distribution of uNK cell density between normal weight and obese women.

5.4 Discussion:

In this immunohistochemical study, I have examined a large number of timed mid-luteal endometrial samples (n=304) from women with various reproductive failures for uNK cell density and have shown that, despite examining a large

number of endometrial biopsies, there is no apparent effect of obesogenic environment on uNK cell density.

There is no significant correlation between the BMI and the uNK cell density, and the distribution of uNK cell density is not significantly different in women of normal, overweight and obese categories. The results also suggest that even in women with a history of recurrent miscarriage, obesity does not appear to affect the peri-implantation uNK cell density

The findings of this study are consistent with the findings of a previous small study (n=28) by Metwally et al., (2007), which investigated the impact of high BMI on endometrial morphology in the peri-implantation period in women with RMC. In their study, the authors analysed various leucocyte populations by immunocytochemistry and showed that uNK cell density ($CD56^+$) was not significantly different in high BMI (BMI > 25, n=16) compared with women of normal weight (BMI < 25, n=12).

My data from this study also shows that an increase in BMI is not associated with any clear effect on the peri-implantation endometrial macrophage density. We have shown that maternal obesity is not associated with significantly greater $CD14^+$ cells within the peri-implantation endometrium compared with normal weight women. In this group of women, there was no correlation between peri-implantation endometrial $CD14^+$ density and maternal age and BMI.

To date, the focus in the literature has been on the immune cell population within the placental villi. Contrasting evidence exists regrding the effect of obesity on macrophage density in the placental villi with some studies suggesting an increase in the resident $CD14^+$ cells by 2-3 fold (Challier et al., 2007; Farley et al., 2009) in the placenta of obese women when compared to non-obese women, whereas the others (Roberts et al., 2012) have found no difference in the the number of $CD14^+$ cells within the placental villi of obese and normal weight women.

Maternal age is an important contributing factor for an increased risk of adverse reproductive outcome in women. Our primary objective was to investigate the leucocyte density in the peri-implantation endometrium, but we acknowledge that the risk of infertility and miscarriages are more with an increase in the maternal age, so we analysed the effect of age on the uterine leucocyte population. In this study (n=299), we did not find any significant difference in the mean (± SD) age between the groups. We also found no significant correlation between maternal age and the uNK cell density (n=299).

These findings are consistent with the findings of previous studies (Clifford et al., 1999; Tuckermann et al., 2007) who found no significant correlation between maternal age and the number of uNK cells in women with early pregnancy RMC. These findings also support the evidence that age related fertility issues are unlikely to originate from the endometrium, but are oocyte related (Abdalla et al., 1993).

One of the greatest strengths of this study is the relatively large sample size in assessing the effects of high BMI on uNK cell density. The endometrial samples were obtained timed to mid-luteal phase (LH+ 5-10 days) which co-incides with an essential endometrial transformation responsible for receptivity and selectivity. The WHO classification of BMI was used to categorise women into different weight groups. The investigator was blinded to the identity of the slide. To our knowledge, this is the first study to address the effects of obesogenic environment on the macrophage population, the second most abundant leucocytes, in the peri-implantation endometrium. To date, the focus in the literature has either been on the macrophage ($CD14^{+}$) concentration in the peri-implantation endometrium in RMC versus control group or on the effect of obesity on the macrophage (CD14+) density within the placental villi. We have also demonstrated the effects of age on the uterine leucocyte population.

We acknowledge that there are a few limitations which need to be taken into account when analysing the results of the study. Firstly, maternal age is an important confounding variable in any study on adverse reproductive outcomes. As described above, maternal age was unlikely to be of any significance for this study.

Secondly, there is a lack of reference technique for assessing the peri-implantation endometrial leucocyte concentration. Different studies have used different methods for the assessment of uNK cell density. Though immunohistochemistry is the method used in most of the studies (Quenby et al.,

1999; Michmata et al., 2002; Tuckerman et al., 2007) as it reveals the location of uNK cells, some studies (Lachapelle et al., 1996; Fukui et al., 1999) have used flow cytometry for uNK cell testing. Quenby et al., (1999) used frozen sections, pressure cooker for antigen retrieval and sub-epithelial region to assess the uNK cell density, whereas Tuckerman et al., (2007) used wax embedded specimens, microwave for antigen retrieval and random counting of uNK cell density including deeper into the section. The uNK cell density can either be counted manually or by digital image analysis and the latter produce more reproducible results (Drury et al., 2011). We used a similar method as described by Tuckerman et al., (2007) for antigen retrieval, but used the method described by Quenby et al., (1999) for manual counting of uNK cell density, but from five random fields in the sub-epithelial region at x400 magnification. Immunohistochemistry has been described for assessing the macrophage density in the peri-implantation endometrium (Quenby et al., 1999; Quack et al., 2001) and a similar method as described above was used for assessing macrophage density in our study.

The number of endometrial biopsies assessed for macrophage density may not have sufficient power to reflect the actual effect of BMI on macrophage density. Analysing the endometrial samples for markers from morbidly obese women (BMI >40) and comparing them with normal weight women with a sufficient power calculation may reflect a better endometrial function.

5.5 Conclusions:

Despite examining a large number of samples from women with various adverse reproductive failure there appears to be no apparent effect of obesity on uNK cell density. The study also suggests that high BMI does not stimulate macrophage accumulation in the peri-implantation endometrium. My findings advocate the concept that obesity, as determined by BMI, is not associated with an adverse peri-implantation endometrial immune environment.

CHAPTER 6:

RELATIONSHIP OF PERI-VASCULAR ENDOMETRIAL MESENCHYMAL STEM CELLS WITH BMI

6.1 Introduction:

As described in Chapter 1, the endometrium is rich in mesenchymal stem-like cells (eMSCs) which are essential for cyclic renewal of the endometrium and uterine function. They are a resident cell population and are recruited to the endometrium in response to hypoxic, proteolytic and inflammatory stimuli associated with cyclic menstruation or pregnancy. W5C5 has been identified as a novel single marker for purifying eMSCs which self-renew, have high clonogenecity and are capable of producing endometrial stroma *in vivo* (Masuda et al., 2012). There have been no studies looking at the effects on BMI on eMSCs in the peri-implantation period.

I hypothesised that the abundance and clonogenic efficiency of endometrial mesenchymal stem-like cells, which play an important role in endometrial regeneration, are impaired in the peri-implantation endometrium of obese women when compared to normal weight women. To address this hypothesis, I set out to analyse the abundance of W5C5+ cells and their cloning efficiency in the peri-implantation endometrium.

6.2 Materials and methods:

Described in chapter 2

6.3 Results:

6.3.1 Demographic details:

Endometrial tissue was obtained from a total of 54 women. The Shapiro-Wilk test for normality suggested that the data for age, % of W5C5+ cells and % of W5C5- cells were normally distributed, whereas the data for BMI, previous livebirths, previous first trimester miscarriages, W5C5+ CE and W5C5- CE did not pass the normality test. The women were classified into three groups according to their BMI: normal (n=22), overweight (n=20) and obese (n=12). The demographic details for each of these groups are in Table 6.1. There was no significant difference in the mean (± SD) age and the number of live births in different weight groups.

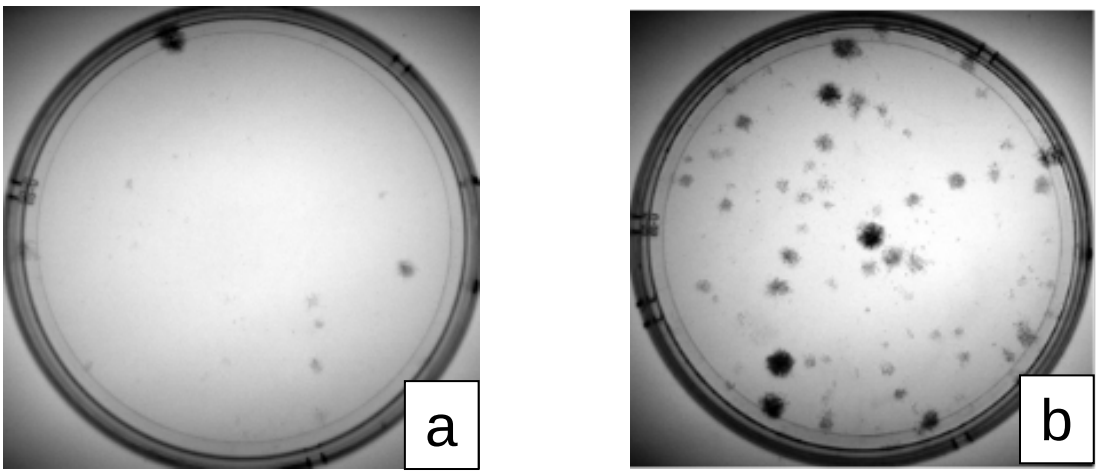


Figure 6.1: Human endometrial cell clones. Freshly isolated W5C5- (a) and W5C5+ (b) cells seeded at 50 cells/cm^2 showing individual colonies.

	Normal (n=22)	Overweight (n=20)	Obese (n=12)	p
Age in years - Mean (± SD)	34.50 (4.48)	36.15 (5.37)	34.67(5.82)	.547##
BMI (Kg/m^2) - Median (IQR)	23 (21.75 - 24)	26 (25 - 28)	32.50 (30.1 – 37.5)	<.0001***#
No of previous Live Births (%)	5 (22.72)	9 (45)	4 (33.33)	0.310###
First Trimester Miscarriage (median ± IQR)	2.0 (0.0 – 3.25)	3.5 (1.0 – 5.0)	6.00 (3.25 – 8.0)	.001#***
W5C5+ cells (%) Mean (± SEM)	8.10 (±.92)	6.96 (±.79)	4.94 (±.87)	.078
W5C5- cells (%) Mean (± SEM)	91.89 (±.92)	93.04 (±.79)	95.05 (±.87)	.078
W5C5+ CE (%) Mean (± SEM)	3.43 (±.62)	2.21 (±.37)	1.04 (±.32)	.013*
W5C5- CE (%) Mean (± SEM)	1.19 (±.35)	.52 (±.19)	.19 (±.07)	.047*

Table 6.1: Summary of demographic, clinical and W5C5 details of the study population. # - Independent samples Kruskal-Wallis nonparametric test; ## - One way ANOVA and post hoc Tukey HSD test; ### - Chi-square test; *** - p=.001; * - p,.05.

Kruskal-Wallis test indicated a significant difference in first trimester miscarriages existed among the three groups (T = 13.01, df=2, p=.001) (Figure 6.2). A pairwise comparison between the groups suggested that the number of previous first trimester miscarriages was significantly higher in obese women compared to normal weight women (p=.001), but no significant difference between the obese-overweight (p=.124) and overweight-normal groups (p=.234) were found (see also chapter 2). BMI was significantly positively correlated with first trimester miscarriages (Spearman's correlation coefficient, rho .493, p=01), but not with live births (Spearman's correlation coefficient, rho .167, p=.228) (Figure 6.3).

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of First Trimester Miscarriages is the same across categories of Groups according to BMI.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Normal-Overweight	-8.466	4.802	-1.763	.078	.234
Normal-Obese	-20.049	5.578	-3.594	.000	.001
Overweight-Obese	-11.583	5.676	-2.041	.041	.124

Figure 6.2: Kruskal-Wallis test indicated a significant difference in the median number of first trimester miscarriages amongst different BMI groups. Pairwise comparison of distribution of first trimester miscarriages indicated that first trimester losses were significantly higher in the obese group when compared to normal weight group (p=.001), but there was no significant differences in the obese compared to overweight group (p=.124) and overweight women compared with normal weight women (p=.234).

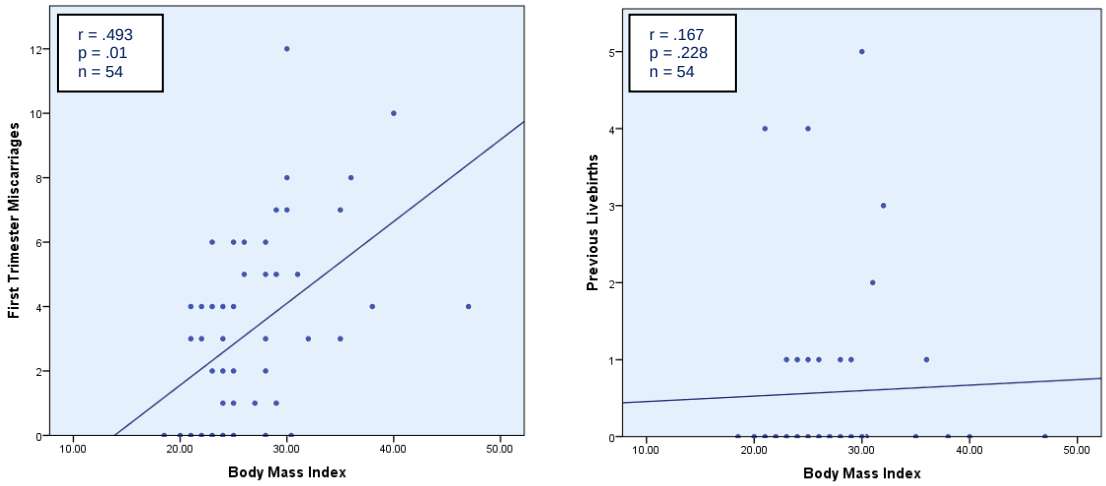


Figure 6.3: Relationship between first trimester pregnancy loss and previous live birth with Body Mass Index in all subjects. First trimister losses are significantly positively correlated with BMI (Spearman correlation coefficient: rho=.493, p=0.01), but live births were not significantly correlated with BMI (Spearman correlation coefficient: rho=.167, p=.228).

6.3.2 The correlation of BMI with % W5C5+ cells, % W5C5- cells, % W5C5+ CE and % W5C5- CE:

Overall, an average of 93% of the cells isolated was W5C5- and 7% were W5C5+. Of the W5C5+ cells, around 2.45% of the cells were clonogenic, whereas only 0.72% of the W5C5- cells were clonogenic.

Relationship between BMI and W5C5+ cell density and W5C5- cell density indicated no significant relationship between the percentage of W5C5+ cells (Pearson correlation coefficient: r = -.243, p=.760) and W5C5- cells (Pearson correlation coefficient: r = .243, p=.760) with BMI (Figure 6.4).

The relative cloning efficiency of both W5C5+ CE (Pearson correlation coefficient: r = -.426, p=.001) and W5C5- CE (Pearson correlation coefficient: r = -.376, p=.005) was significantly negatively correlated with the BMI (Figure 6.5)

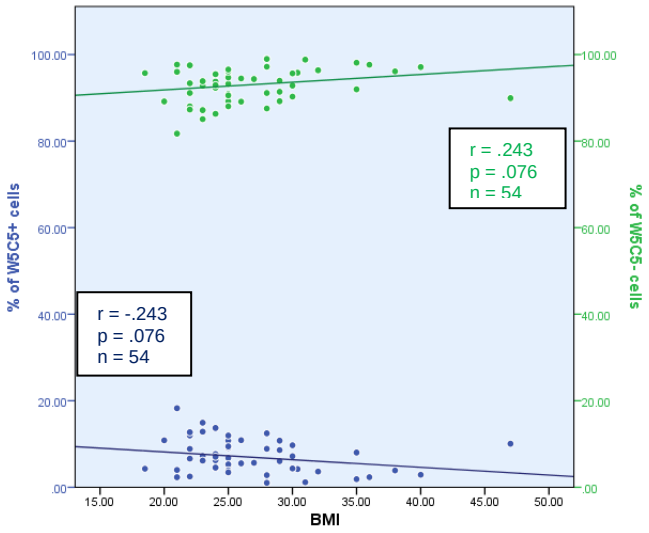


Figure 6.4: Relationship between % of W5C5+ cells and % of W5C5- cells with BMI. No significant correlation was observed between the percentage of W5C5+ cells (Pearson correlation coefficient: r = -.243, p=.760) and W5C5- cells (Pearson correlation coefficient: r = .243, p=.760) with BMI.

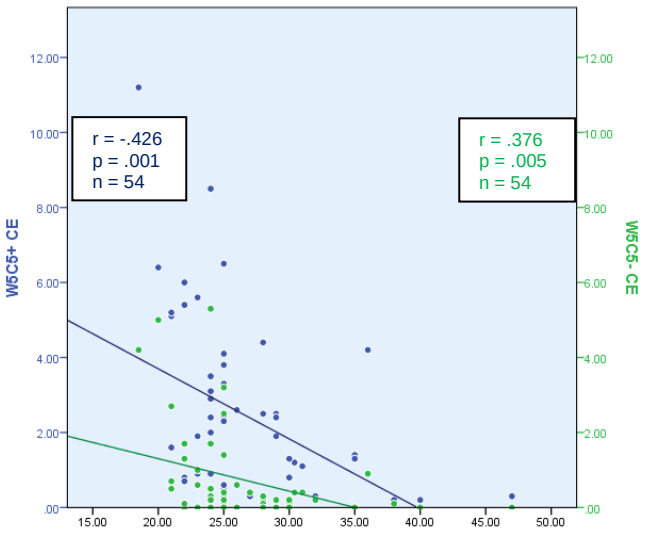
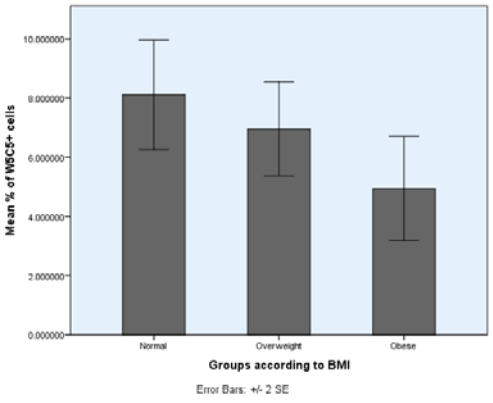


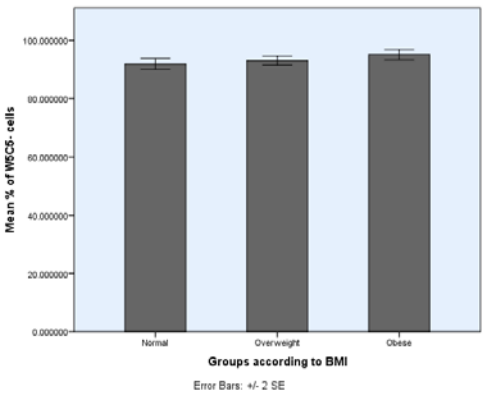
Figure 6.5: Relationship between % of W5C5+ CE and % of W5C5- CE with BMI. The relative cloning efficiency of both W5C5+ CE (Pearson correlation coefficient: r = -.426, p=.001) and W5C5- CE (Pearson correlation coefficient: r = -.376, p=.005) was significantly negatively correlated with the BMI.

6.3.3 Effect of BMI on % W5C5+ cells, % W5C5- cells, % W5C5+ CE and % W5C5- CE:

One way ANOVA test was carried out to compare the % of W5C5+ and % of W5C5- cells among the three groups which indicated no significant differences in the relative (%) abundance of W5C5+ cells (p=.078) and % of W5C5- cells (p=.078) between obese, overweight and subjects with normal BMI (Figure 6.6). One way ANOVA and post-hoc Tukey's HSD test indicated that % W5C5+ CE was significantly lower in obese subjects compared to subjects with normal BMI (p=0.01), but there was no significant difference between overweight-normal (p=.18) and obese-overweight groups (p=.320). One way ANOVA suggested a significant difference in % W5C5- CE among the groups, but post-hoc Tukey's test showed a trend towards decreased CE in obese women when compared to normal weight women, but was not statistically significant (p=.057) (Figure 6.7).

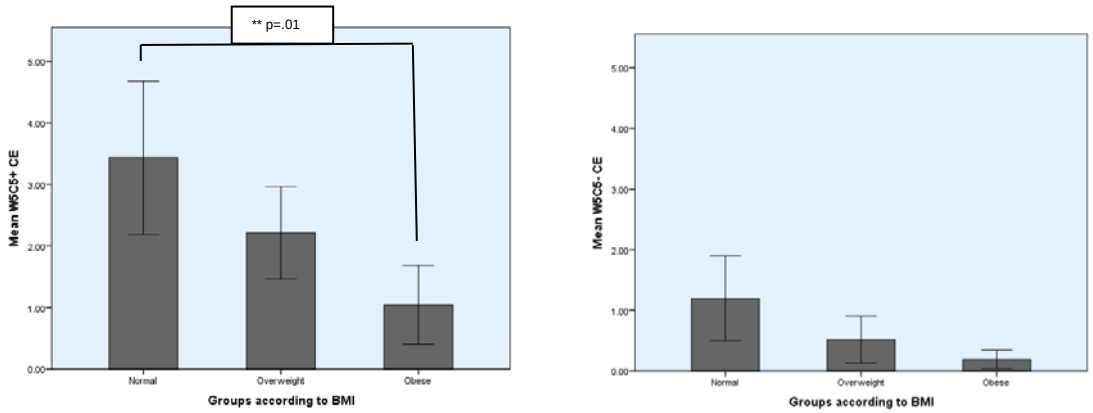


a.



b.

Figure 6.6: (a) W5C5+ cells (%) and (b) W5C5 – cells (%) were similar in all BMI groups. Data in mean ± SEM. Group comparison by one way ANOVA and post hoc Tukey's HSD test.



a. b.

Figure 6.7: % W5C5+ CE (a) was significantly less in obese women when compared to women with normal BMI. No significant difference in the % W5C5 – CE (b) when compared in different BMI groups. Data in mean ± SEM. Group comparison by one way ANOVA and post hoc Tukey's HSD test.

6.3.4 Effect of age on % W5C5+ cells, % W5C5- cells, % W5C5+ CE and % W5C5- CE:

We analysed the relationship between maternal age and the % W5C5+, % W5C5-, % W5C5+ CE and % W5C5- CE. The women were classified into three groups according to their age: less than 35 years (n=22), 35—39.9 years (n=20) and more than 40 years (n=12). There was no significant difference in the median (± IQR) BMI in different age groups.

Relationship between BMI and W5C5+ cell density and W5C5- cell density indicated no significant correlation between W5C5+ cell density (Spearman's

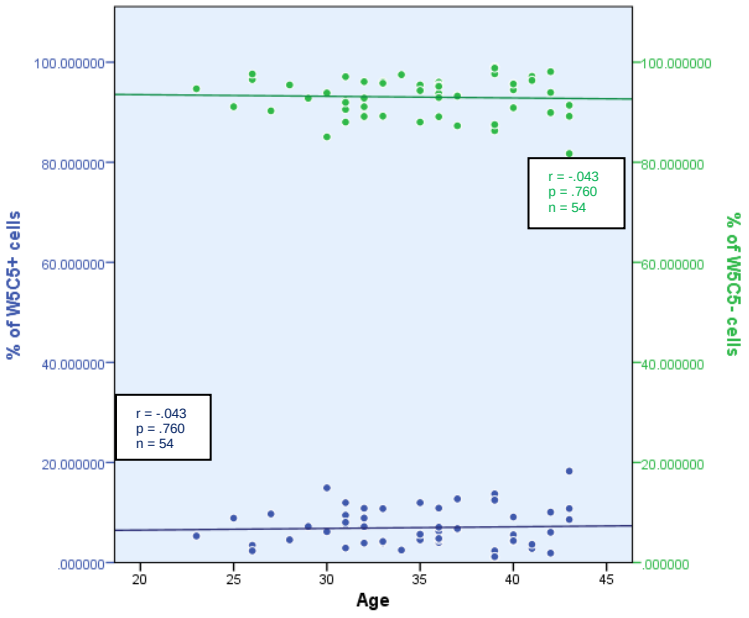
correlation coefficient, rho -.065, p=.641) and W5C5- cell density (Spearman's correlation coefficient, rho -.022, p=.874) with age.

	< 35 years (n=22)	**36 – 39 years (n=20)**	**40 years (n=12)**	**p**
BMI (Kg/m^2) - Median (IQR)	25 (23.0 – 30.1)	24 (23.25 – 26.75)	28.50 (25.0 – 31.5)	.138#
W5C5+ cells (%) Mean (± SEM)	7.03 (±.73)	6.84 (±.94)	7.11 (±1.32)	.980##
W5C5- cells (%) Mean (± SEM)	92.96 (±.73)	93.15 (±.94)	92.88 (±1.32)	.980##
W5C5+ CE (%) Mean (± SEM)	2.62 (±.44)	2.24 (±.51)	2.47 (±.89)	.871##
W5C5- CE (%) Mean (± SEM)	.77 (±.26)	.68 (±.28)	.70 (±.39)	.970##

Table 6.2: Summary of demographic, clinical and W5C5 details of the study population according to age. # - Independent samples Kruskal-Wallis nonparametric test; ## - One way one way ANOVA and post hoc Tukey HSD test.

No significant relationship was observed between the % of W5C5+ cells (Pearson correlation coefficient: r = .043, p=.760), % of W5C5- cells (Pearson correlation coefficient: r = -.043, p=.760) and the relative cloning efficiency of both W5C5+ CE (Pearson correlation coefficient: r = -.147, p=.288) and W5C5- CE (Pearson correlation coefficient: r = -.085, p=.543) with age (Figure 6.8).

a.



b.

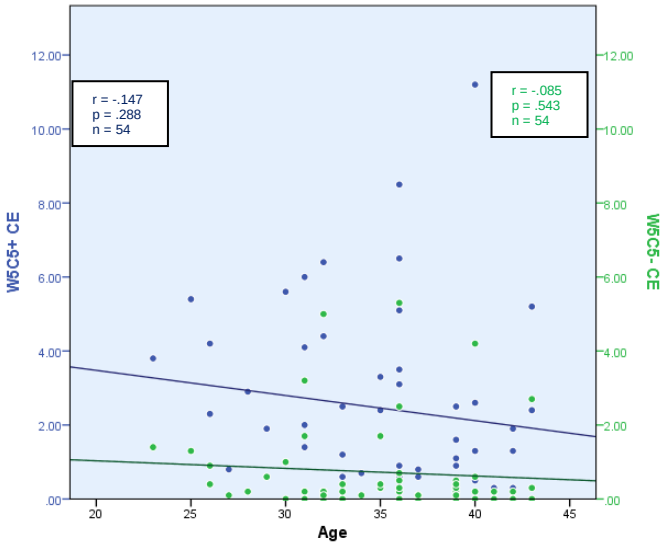


Figure 6.8: a) Relationship between % of W5C5+ cells and % of W5C5- cells with age. No significant correlation was observed between the percentage of W5C5+ cells (Pearson correlation coefficient: r = .043, p=.760) and W5C5- cells (Pearson correlation coefficient: r = -.043, p=.760) with BMI. b) Relationship between % of W5C5+ CE and % of W5C5- CE with BMI. No significant correlation was observed between the cloning efficiency of both W5C5+ CE (Pearson correlation coefficient: r = -.147, p=.288) and W5C5- CE (Pearson correlation coefficient: r = -.085, p=.543) with age.

One way ANOVA test was carried out to compare the relative cell density and CE among the three groups, which indicated no significant differences in the relative (%) abundance of W5C5+ cells (p=.980), % of W5C5- cells (p=.980) % W5C5+ CE (p=.871) and % W5C5- CE (p=.970) between the three age groups (Figure 6.9).

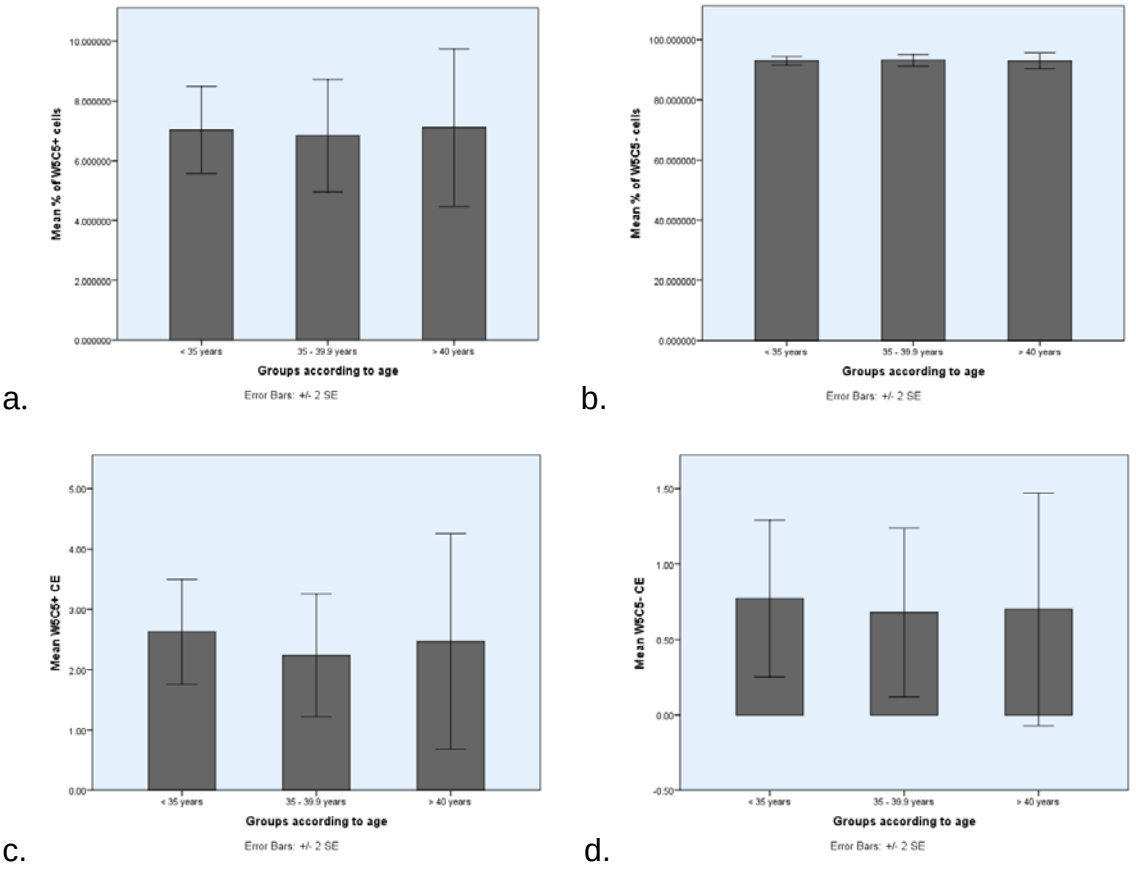


Figure 6.9: Group comparison of W5C5+ cell density, W5C5- cell density, W5C5+ cells (%), W5C5 – cells (%), W5C5+ CE and W5C5 – CE; a) Results of the Kruskal-Wallis test indicated that there was no significant difference in the W5C5+ cell density (T = 2.690, df=2, p=.261) in all age groups, data in median ± IQR. b) Results of the Kruskal-Wallis test indicated that there was no significant difference in the W5C5- cell density were similar in all age groups (T = 5.655, df=2, p=.059), data in median ± IQR. One way ANOVA test indicated no significant differences the relative (%) abundance of W5C5+ cells (c) (p=.980), (d) % of W5C5- cells (p=.980), (e) % W5C5+ CE (p=.871) and (f) % W5C5- CE (p=.970) between the three age groups, data in mean ± SEM.

6.4 Discussion:

In this study I have demonstrated that the cloning efficiency of W5C5+ (eMSCs) was significantly reduced in obese subjects when compared to normal weight subjects. I also found a significant negative correlation of CE of W5C5+ and W5C5- cells with BMI. My study did not find any significant impact of obesity on the abundance of W5C5+ cell density and W5C5- cell density in women suffering reproductive failures.

My study findings from this group of women has demonstrated again that in women with increasing in BMI has a significant positive correlation with a history of increased number of first trimester miscarriages (discussed in detail in chapter 2).

Clonogenecity (CE) is a remarkable feature of eMSCs which confers incredible regenerative capacity to the human endometrium. We demonstrated that the cloning efficiency of both W5C5+ and W5C5- cells was significantly negatively correlated with the BMI with a significant decreased CE of W5C5+ cells in obese women when compared to normal weight women.

These findings from my study suggest that the regenerative capacity and the plasticity of the endometrium may be suboptimal in obese women when

compared to that of normal weight women. This may potentially compromise the ability of the uterus to undergo intense tissue remodelling upon embryo implantation, thus increasing the risk of miscarriage and other pregnancy complications in obese women. However, it is important to acknowledge that other mechanisms, such as a chronic pro-inflammatory state associated with obesity, may also contribute to the increased risk of adverse reproductive outcomes (Denison et al., 2010). The clinical relevance of suboptimal CE of W5C5+ cells in an obesogenic environment is reinforced by the observation that the obese subjects had higher miscarriage rates when compared to either overweight or normal women.

As discussed in previous chapters, maternal age is an important factor in determining successful pregnancy outcome. The risk of miscarriages and infertility significantly increase with advancing maternal age. However, this study did not find any significant differences in the abundance or CE of W5C5+ and W5C5− endometrial cells between the three age groups (less than 35 years, 35-39.9 years and more than 40 years). Additionally, there were no significant correlations between the abundance and CE of W5C5+ and W5C5− cells and age. These are key findings which agree with the recognised concept that the age related decline in fertility and poor reproductive outcomes are likely to be associated with an ovarian disorder rather than endometrial dysfunction.

My work could potentially translate into novel diagnostic (e.g. quantifying and characterizing W5C5+ and W5C5- endometrial cells) interventions for

reproductive failure. Further studies are required to understand the defined mechanisms that govern the activity of endogenous eMSCs and the recruitment of clonogenic cells into the dynamic endometrium.

This is the first prospective study to present a significant association between W5C5 endometrial cells and their CE with BMI, age and reproductive outcome. The other strength of this study is that it was carried out specifically to address our hypothesis.

A limitation of our study lies in the relatively small number of subjects studied. A further potential criticism is that we did not examine the cell density and CE of the cells from a control group i.e. women with no adverse pregnancy outcome. The study was performed on endometrial biopsies obtained from women in the mid-luteal phase as required for us to study other markers in the endometrium which has limited our understanding of the effect of BMI on these cells and their function in different phases of the menstrual cycle. Further studies looking at these two factors on eMSCs in adequately powered prospective studies would certainly be of interest.

6.5 Conclusions:

The clonogenic eMSCs which are capable of producing endometrial stroma *in vivo* allow the human endometrium to display remarkable regenerative capacity and plasticity. Our observations suggest that these essential features of the endometrium may be suboptimal in obese women which may be responsible for the increased risk of reproductive complications associated with obesity. Our data provides a platform for future studies on endometrial stem cell research.

CHAPTER 7:

THE EFFECT OF BMI ON DIFFERENTIATING ENDOMETRIAL STROMAL CELLS *IN VITRO*

7.1 Introduction:

As described in detail in chapter 1, decidualisation is a process that involves endometrial stromal cell differentiation into specialized secretory decidual cells, altered local immune cell populations and remodelling of the spiral arteries (Brosens et al., 2002) and the endometrium acquires distinctive characteristics essential for pregnancy. Decidualisation is essential for the formation of the placenta by its ability to regulate trophoblast invasion, and to maintain homeostasis by resisting environmental and oxidative stress. Endometrial stromal cells (ESC) modulate local vascular and immune responses and act as biosensors of embryo quality upon decidualisation (Teklenberg et al., 2010). Impaired cyclic decidualisation disables natural embryo selection and predisposes to recurrent pregnancy failures (Salker et al., 2010). Differentiation of ESCs in primary cell cultures *in-vitro* reflects decidualisation *in-vivo* and can be achieved by stimulation of ESCs using cyclic AMP – a second messenger molecule, and a progestin – medroxyprogesterone acetate (MPA) (Brosens et al., 1999). Expression of decidualisation markers, prolactin (*PRL*) and Insulin like growth factor binding protein–1 (*IGFBP1)* can be measured using real time quantitative PCR analysis.

Adipose tissue synthesizes and secretes several adipokines - proteins and bio active polypeptides, and hormones which are important not only for maintaining energy homeostasis, but can also influence reproduction by their action on various reproductive organs including the endometrium (Michalakis and Segars,

2010). Adipokines act as systemic inflammatory mediators making obesity a state of low-grade inflammation. The fat within the organs and in the peri-vascular region also has significant metabolic effects (Lee et al., 2009). Obese pregnant women are found to have an increased systemic and placental inflammation and associated endocrine and immune functions (Stewart et al., 2007; Challier et al., 2008). Should the chronic inflammation associated with obesity affect the peri-implantation endometrium of high BMI women, this may have implications for endometrial stromal decidualisation and subsequent placental development and thus on potential fertility and reproductive outcome.

We recognise that decidualisation of ESCs is an important step for successful implantation and pregnancy, so we sought to investigate if obesity could have a deleterious effect on the endometrial support of early pregnancy. We studied the effect of obesity on the peri-implantation endometrium by utilizing an *in-vitro* model of endometrial stromal cell decidualization. There are no studies which looked at the effect of obesity on the ESC *differentiation in vitro.*

I hypothesised that the decidual programming of the peri-implantation endometrium is perturbed in an obesogenic environment and set out to assess the decidualisation markers from differentiating ESCs obtained from normal weight and obese women. I also looked at differentiation of ESCs in artificially created obesogenic environment.

7.2 Materials and methods:

As discussed in chapter 2

7.3 Results:

7.3.1 Demographic details:

Endometrial tissue was obtained from a total of 12 women. The Shapiro-Wilk test for normality suggested that the data for BMI was normally distributed, whereas the data relative expression of *IGFBP1* on day 4, relative expression of *IGFBP1* on day 8, relative expression of *PRL* on day 4, relative expression of *PRL* on day 8 did not pass the normality test. When the data for above variables were transformed into $\log^{10}$ values, the Shapiro-Wilk test indicated that the data was normally distributed. The women were classified into two groups according to their BMI: BMI < 25 (n=6) and BMI > 25 (n=6) (Figure 7.1).

	BMI < 25 (n=6)	BMI > 25 (n=6)	p
BMI (Kg/m^2) Mean (± SEM)	22.87 (.452)	31.83 (1.81)	<.002**#
Age in years Median (IQR)	34 (31.5 – 36.0)	34.50 (30.75 – 43.0)	.699##
Relative induction of *IGFBP1 mRNA* day 4 Mean (± SEM)	177.67 (± 96.23)	1538.05 (± 1514.19)	.937#
Relative induction of *IGFBP1 mRNA* day 8 Mean (± SEM)	233.33 (± 144.19)	2092.08 (±2031.70)	.394#
Relative induction of *PRL mRNA* day 4 Mean (± SEM)	912.20 (± 416.60)	510.67. (± 287.45)	.485#
Relative induction of *PRL mRNA* day 8 Mean (± SEM)	2013.21 (± 826.75)	1998.27 (± 1161.02)	.937#

Table 7.1: Summary of BMI and relative expression of decidualisation markers. # - Independent samples T test; ## - Independent samples Mann-Whitney U test; ** - *p=.001*. There was no significant difference in the relative expression of both *IGFBP1* and *PRL* on day 4 and day 8 in both groups.

	BMI < 25 (n=6)	BMI > 25 (n=6)	p
Log^{10} relative induction of *IGFBP1 mRNA* day 4 Mean (± SEM)	1.06 (± .69)	1.05 (± .70)	.997#
Log^{10} relative induction of *IGFBP1 mRNA* day 8 Mean (± SEM)	.96 (± .69)	1.80 (± .56)	.366#
Log^{10} relative induction of *PRL mRNA* day 4 Mean (± SEM)	2.65 (± .29)	2.09 (± .37)	.255#
Log^{10} relative induction of *PRL mRNA* day 8 Mean (± SEM)	2.94 (± .34)	2.68 (± .38)	.610#

Table 7.2: Summary of Log^{10} relative induction of decidualisation markers on day 4 and day 8. # - Independent samples T test with equal variances assumed.

7.3.2 Effect of BMI on expression of decidualisation markers in differentiating ESCs:

Independent samples Mann-Whitney U test indicated no significant difference in the relative expression of *IGFBP1* on day 4, relative expression of *IGFBP1* on day 8, relative expression of *PRL* on day 4, relative expression of *PRL* on day 8 among the two groups (Table 7.1 and Figures 7.1 a & b). The relative induction of decidualisation markers were logarithmically transformed into Log^{10} variables

(Table 7.2) and an independent samples T test with equal variances assumed indicated no significant difference in the median relative expression of *IGFBP1* and *PRL* on days 4 and 8 amongst both BMI groups (Figures 7.1 c & d).

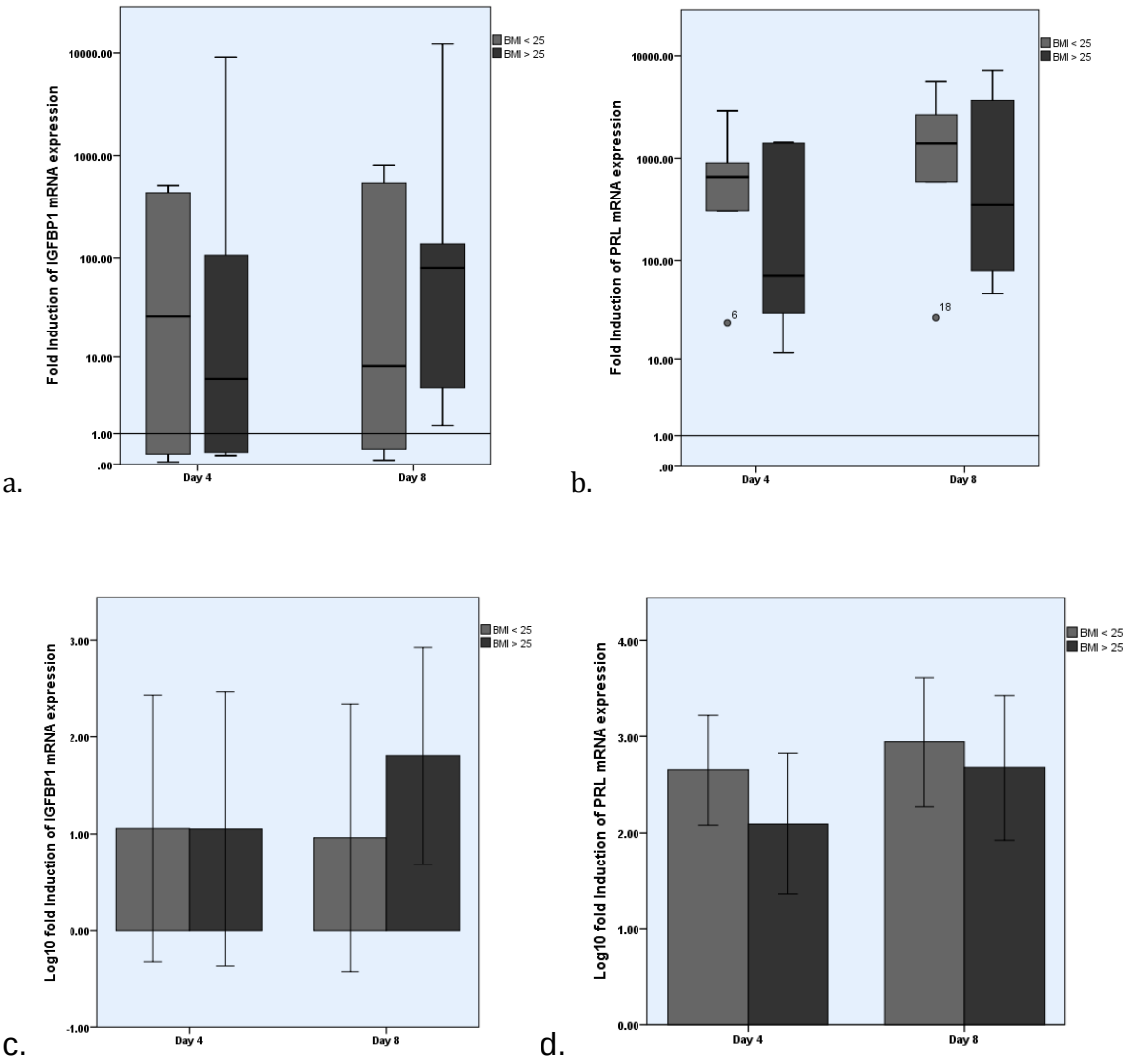


Figure 7.1: Box and Whisker plots showing the results of Independent samples Mann-Whitney U test indicated no significant difference in the median relative expression of *IGFBP1* and *PRL* on days 4 and 8 amongst different BMI groups (a) and (b). Data in median (± IQR). Note the logarithmic Y-axis. The Bar chart showing the above data logarithmically transformed into Log10 data and an independent samples T test results which indicated no significant difference in the relative expression of *IGFBP1* and *PRL* on days 4 and 8 amongst both BMI groups (c) and (d). Data in mean ± SEM

7.3.3 Effect of supernatant from fat explants on expression of decidualisation markers in differentiating ESCs:

The earlier experiments indicated that maternal characteristics, particularly the BMI, did not affect the differentiation of hESCs in-vitro. We hypothesized that absence of the obesogenic environment at the time of decidualisation to be the reason for this behaviour of hESCs. We aimed to create an artificial obesogenic environment in-vitro at the time of differentiation of hESCs to investigate the possible effects of metabolically active substances secreted by an excess adipose tissue on endometrial stromal compartment decidualisation. We obtained supernatant from adipose tissue explants from normal weight women and obese women and treated them to the differentiating ESCs for 24 hours.

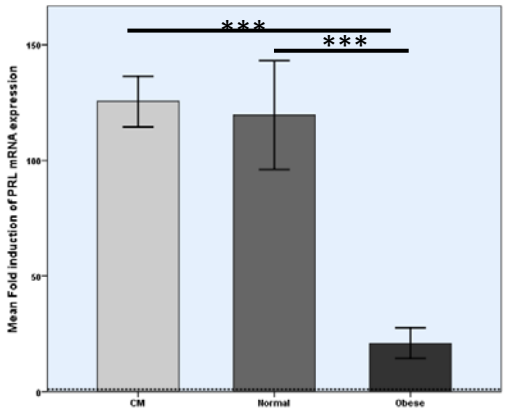
The results obtained indicated that the *PRL* expression was significantly inhibited in hESCs differentiating in supernatant from the adipose tissue explants of obese woman when compared to the other groups.

Figure 7.2 (a) and table 7.3 demonstrate that there was a significant difference in the expression of *PRL* between the groups (F=50.15, df=2, p=.000). A Tukey's HSD post-hoc test revealed that the *PRL* expression was significantly lower in the obese group when compared to CM group (p=.001) and also from the normal group (p=.001).

Figures 7.2 (b) and table 7.3 indicate that the *IGFBP1* expression was significantly lower between the groups (F=7.00, df=2, p=.035). A multiple

comparison post-hoc Tukey HSD test indicated no significant difference in the *IGFBP1* expression between normal-CM and normal-obese groups, but a statistically significant difference between the obese group when compared to CM group (p=.032).

a.



b.

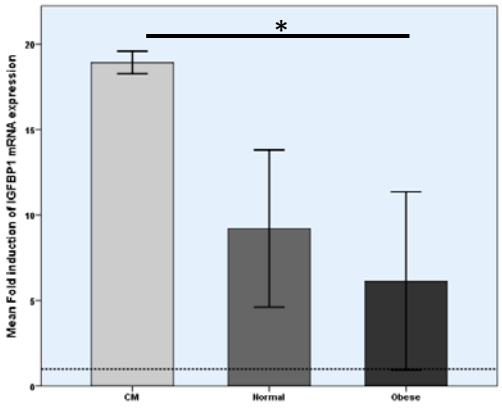


Figure 7.2: Bar charts with error bars indicating the *PRL* and *IGFBP1* transcript levels in timed endometrial biopsies treated with culture media (CM), supernatant from adipose tissue explants from normal weight women (Normal) and obese women (Obese). *PRL* (a) and *IGFBP1* (b) *mRNA* levels normalised to *L19* transcript levels. Data in mean ± SEM. *** - *p=.001* and * - *p=.032.*

a.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Fold induction of PRL mRNA expression	Between Groups	19146.810	2	9573.405	50.150	.000
	Within Groups	954.476	5	190.895		
	Total	20101.286	7			
Fold induction of IGFBP1 mRNA expression	Between Groups	203.839	2	101.919	7.006	.035
	Within Groups	72.738	5	14.548		
	Total	276.577	7			

Table 7.3: Results of oneway ANOVA for *PRL* and *IGFBP1* expression in decidualising hESCs treated with culture media (CM), supernatant from adipose tissue explants from normal weight (Normal) and obese (Obese) women

7.4 Discussion:

My study findings provide evidence for the first time that the differentiation of human endometrial stromal cells in-vitro is not affected by the BMI of the women. This is based on the observation that expression of *PRL* and *IGFBP1*, highly sensitive and specific decidual marker genes within the endometrium, is not impaired in primary cell cultures obtained from women of different categories, when they are exposed to a decidualising stimulus. The levels of these marker genes were significantly not different in the timed endometrial biopsies from women with a normal weight (BMI < 25) when compared to the endometrial samples obtained from overweight and obese women. Although our expression analysis was restricted only to *PRL* and *IGFBP1* expression, the data has demonstrated that the maternal characteristics such as BMI does not have any significant effect on the differentiating stromal cells *in-vitro*.

However, this study has also provided an interesting evidence that the decidual programming may be impaired in an obesogenic environment. The levels of *PRL* were significantly lower in the decidualising endometrial stromal cells treated with supernatant from adipose tissue explants obtained from obese women when compared to cells treated with either the plain culture media or the supernatant from adipose tissue explants obtained from normal weight women. The treatment of decidualising endometrial stromal cells to supernatant from adipose tissue explants obtained from normal weight women did not make any significant effect on the expression of IGFBP1 expression when compared to

cells treated with plain culture media, whereas, the addition of supernatant from adipose tissue explants obtained from obese women to decidualising endometrial stromal cells significantly inhibited IGFBP1 when compared to the cells treated with culture media only. These are important findings which indicate that the metabolically active substances in the adipose tissue from obese women exert an unfavourable effect on the endometrial preparation for embryo implantation and pregnancy.

Previous studies have demonstrated that the perturbations in decidual transformation of the endometrial stromal compartment can have negative effects on the endometrial programming in preparation of pregnancy and can lead to poor reproductive outcome such as infertility, miscarriage and RMC (Salker et al., 2010; Salker et al., 2011). The PRL expression was demonstrated to be significantly impaired in decidualising hESCs from women with RMC when compared to decidualising cells from control women (Salker et al., 2010). Our study demonstrates that impaired decidualisation of resident stromal cells in an obesogenic environment may predispose to unfavourable reproductive outcomes by disabling embryo recognition and by disrupting maternal responses to embryonic signals. In our observational study, we have indeed demonstrated that obesity is associated with increased risk of miscarriage of early pregnancy (discussed in chapter 2). An associated shorter time to pregnancy intervals in obese women with RMC (as discussed in chapter 2) could also be explained by the impaired decidualisation leading to impaired endometrial receptivity and natural embryo selection that even allows delayed

implantation of developmentally compromised embryos and subsequent pregnancy loss.

The strength of this study is that these findings are novel as hitherto there is no data outlining the effects of obesity on decidual programming of the endometrium which is an important mechanism that determines reproductive success.

There are a few limitations to this study. Firstly, the adipose tissue samples to mimic the obesogenic environment were obtained from women undergoing caesarean section at term, and hence may not account for the gestational variations in the component and metabolic function of the adipose tissue. The adipocytokines, synthesized and secreted by adipose tissue, are a novel group of endogenous substances and the exact mechanism by which these substances cause their effect on the decidualising endometrium is not known. This is an area with potential for future research and therefore, future studies should be undertaken to address this point. The findings are all obtained from *in vitro* cultures of hESCs mainly concentrating on two decidualisation markers and hence may not strictly reflect the in vivo situation and hence, caution is needed in translating our findings to the clinical scenario. There are many other markers such as cytokines (Prokineticin-1; PROK1), regulators (SGK1, IL-33) and pro-inflammatory mediators (ST2) which are linked to decidualisation and further study to assess the expression of these markers would be required for a better understanding of the effects of obesity on decidualisation.

7.5 Conclusions:

This study provides exciting novel data on the effects of the obesogenic environment on the decidual programming of the endometrium. Although, from our in-vitro study, we conclude that the effects of obesity may impair endometrial decidualization and could account for the unfavourable reproductive outcomes associated with obesity. Further work, however, is necessary to confirm these findings in larger studies and also to ascertain the exact underlying mechanisms which explain the metabolic effects of obesity on decidualisation. If confirmed, the findings present us with a novel therapeutic target to improve reproductive outcomes in obese women and suggest that improving decidualization may be of primary importance.

CHAPTER 8:

SUMMARY OF FINDINGS AND CONCLUSIONS

In this thesis, firstly I have studied the clinical issue of the effects of obesity on fecundity and early pregnancy outcome from a large number of women attending a tertiary referral clinic with various reproductive failures and secondly, I have described the peri-implantation endometrium in normal and obese women by utilizing an in-vitro model which has provided a better understanding of uterine leucocytes, mesenchymal stem cells and stromal cell differentiation in the obese micro-milieu. From this study some interesting outcomes and significant differences amongst the study groups were identified which have generated further questions to future research. The findings of my research are summarised below:

I have studied the current evidence on the effects of obesity on female reproductive health particularly looking at fertility, infertility treatment and miscarriage. The evidence from these studies, mainly observational and retrospective, suggests that obese women may be at an increased risk of adverse early pregnancy complications. I have also analysed and presented the clinical evidence on the possible effects of obesity on ovarian function, embryo development and the role of endometrium. There is a dearth of good quality evidence to conclude whether the metabolic effects of obesity are utero-centric or egg-centric, but it is likely that the endometrium may be most affected by obesity.

In chapters 3 and 4, the clinical data identified supporting evidence superfertility existed in RM population and that superfertility was more prevalent amongst

obese than in normal weight women with a history of RM. I also identified that obesity increases the risk of first trimester miscarriages both in general population and in women susceptible to RM and that the obese women had an increased number empty gestational sac pregnancy losses (karyotypically normal - Morikawa et al., 2004) compared to non-obese women in general population, but not in RM group. Both these findings and the available evidence suggest that in obese general population, the empty gestational sac losses are less likely to be karyotypically abnormal i.e. not due to oocyte abnormalities. Whereas the obese women with a history of RM when compared to normal weight counterparts, were more superfertile, and did not have a significant difference in the pregnancy loss pattern. This finding suggests of a possible impaired natural embryo 'quality control' process of the peri-implantation endometrium which allows both developmentally competent as well as compromised embryos to implant, but end up clinically as a miscarriage.

Studies suggest that obesity is characterised by a local adipose tissue inflammation and reactive peripheral leucocytosis (Herishanu et al., 2006). An increased uNK cell density and decidual macrophages density has been reported in the endometrium of women with RMC (Lachapelle et al., 1996; Quenby et al., 1999, 2005, 2009; Tuckerman et al., 2007). However, my study as described in chapter 5, did not find any correlation between BMI and uterine leucocyte density in the immunohistochemical study of mid-luteal endometrial samples. I found no significant difference in the uNK cell density and macrophage density and any significant difference in the ratio of uNK cells to macrophage density in obese women when compared to normal weight women.

Further studies with larger numbers are warranted to investigate the correlation between macrophage density and first trimester miscarriages observed in our study and its significance. Hence, I conclude that the endometrial dysfunction in obese women with reproductive failure does not appear to be immune cell mediated.

In chapter 6, I have demonstrated for the first time the negative impact of obesity on the regenerative capacity and plasticity of the endometrium. Clonogenic W5C5+ cells which are specialised eMSCs that characterise the human endometrium with an extraordinary regenerative capacity. There was no significant correlation between the relative abundance (%) of W5C5+ to W5C5- cells with BMI and no significant difference % of W5C5+ cells among BMI groups. The most interesting findings in this chapter were that of the negative correlation of CE of W5C5+ cells with BMI and the significantly reduced CE of W5C5+ cells in obese subjects when compared to normal weight subjects. Thus obesity appeared to have a deleterious effect on the clonogenecity of endometrial stem cells which is likely to be translated into endometrial dysfunction that the clinical data suggests.

In chapter 7, I have demonstrated that obesogenic environment may possibly impair decidual programming of the endometrial stromal compartment, an essential mechanism that determines the reproductive success in the obesogenic environment. Initial data suggested that the in-vitro decidualisation of the human endometrial stromal cells based on the observation of expression

of decidualisation markers was not affected by the BMI. However, when decidualising endometrial stromal cells were treated with supernatant from adipose tissue explants obtained from obese women, the PRL expression was significantly inhibited when compared to the treatment with supernatant from adipose tissue explants obtained from normal weight women and also when treated only with culture media. The expression of the other marker, IGFBP1 was significantly inhibited when decidualising endometrial stromal cells were treated with supernatant from adipose tissue explants obtained from obese women compared to the cells treated only with culture media. The exact mechanism for inhibition of decidualisation markers in the obese micro-milieu needs further investigation.

In conclusion this work has provided initial evidence to the understanding of the peri-implantation endometrium in obese women. My data suggests that endometrial stromal function is possibly modulated by metabolic influences and that the increased risk of poor reproductive outcome in obese women has a molecular basis that lies in the endometrium. Future work with larger numbers and in-vivo studies in animal models are required to confirm these findings. If confirmed, clinical trials with therapeutic targets to improve decidualisation and thus reproductive outcome in obese women could hold significant clinical potential.

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